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VOLUME 3 FEBRUARY 1957 NUMBER 1

# A NUTRITIONAL STUDY OF THREE STRAINS OF CLAVICEPS PURPUREA (FR). TUL.[1]

W. A. TABER AND L. C. VINING

## Abstract

Organic nitrogen sources were more readily utilized than inorganic nitrogen sources by three strains of *Claviceps purpurea*. Glucose, fructose, mannose, cellobiose, and sucrose were readily utilized, but not xylose, ribose, mannitol, maltose, starch, powdered cellulose, carboxymethyl cellulose, or methyl cellulose. Carbon dioxide was required for initiation of growth. An exogenous supply of biotin was required for growth in a medium containing glucose, ammonium succinate, and minerals. This requirement could be nearly replaced by equimolar amounts of biotin L-sulphoxide and biocytin. Desthiobiotin slightly replaced biotin but homobiotin, norbiotin, oleate, aspartate, pimelate, and Tween 80 were inactive. Oleate and aspartate stimulated early growth.

## Introduction

For a number of years attempts have been made to induce the fungus *Claviceps purpurea* to synthesize ergot alkaloids in pure culture. These have been singularly unsuccessful, except in the case of Abe and co-workers (1, 2, 3, 4), who have recently reported the identification of mixtures of the alkaloids from cultures grown on liquid media. Although the production of the commercially important alkaloid, ergometrine, appeared to be very small, strains were found which, under the same conditions, produced substantial amounts of new and hitherto unknown members of the ergot alkaloid family. Stoll and co-workers (24), adapting the conditions employed by the Japanese workers, have also obtained good yields of a new alkaloid from a species of *Claviceps*. Long periods of incubation seem to be important for the appearance of alkaloids in the mycelium and culture broth, but the pH and composition of the media also play a significant role. The selection of a mannitol – ammonium succinate medium buffered with succinic acid to pH 5.2 may also have been responsible for the success achieved by Abe and his group.

The nutrition of *Claviceps purpurea* has been studied by numerous workers (Abe *et al.* (1), Gjerstad and Ramstad (9), McCrea (16), Michener and Snell

[1]Manuscript received September 7, 1956.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
Issued as N.R.C. No. 4161 and as Paper No. 235 on the Uses of Plant Products.



[The previous number of Can. J. Microbiol. (2, 623–763) was issued December 21, 1956.]

(18), Paul *et al.* (19), Robbins and Ma (22), and Tyler and Schwarting (27)) but considerable disagreement in the results reported suggests that utilization of substrates is markedly affected by either differences in strain or the cultural conditions employed. Since these results could not be applied to our own investigations with confidence, a study of the nutrition of *Claviceps purpurea* was initiated, and three strains of different geographical origin were compared.

## Materials and Methods

### *Cultures*

Three cultures were studied. Two of these, a Spanish strain (II), PRL 1555, and a Portuguese strain, PRL 1556, were obtained from the Centraalbureau voor Schimmelcultures, Baarn. The third culture, PRL 1557, was isolated from sclerotia collected near Saskatoon in 1955. The authenticity of these cultures was sought by packing the flowers of rye plants grown in the greenhouse with vegetative mycelia and noting the appearance of sclerotia or honeydew. PRL 1557 produced abundant sclerotia and honeydew, PRL 1556 abundant honeydew, and PRL 1555 gave no indication of parasitism. Testing of the latter is being continued.

### *Inoculum*

The stock cultures were maintained on a medium having the following composition: glucose, 1 g.; soluble starch, 2 g.; Bacto peptone, 0.5 g.; yeast extract, 100 mg.; $MgSO_4 \cdot 7H_2O$, 100 mg.; $CaCl_2 \cdot 2H_2O$, 50 mg.; $FeSO_4 \cdot 7H_2O$, 0.02 mg.; $ZnSO_4 \cdot 7H_2O$, 0.02 mg.; $M/15$ monobasic phosphate, 40 ml.; $M/15$ dibasic phosphate, 60 ml.; agar, 15 g.; distilled water, 1 liter. Transfers of the strains were made to this medium immediately after their receipt or isolation and these initial stock cultures were used to prepare the inocula throughout the study. The mycelia to be used in the inoculation of the experimental media were prepared by inoculating, with fragments from the stock cultures, 70 ml. of a medium containing 1% glucose and 1% yeast extract. Following incubation for 7 days on a rotary shaker, the mycelia were washed with physiological saline by decantation, homogenized in a Waring blendor, and repeatedly rewashed with saline by centrifugation. With few exceptions the inoculum was used the day it was prepared.

### *Preparation of Media*

The composition of the basal medium employed in the succeeding experiments was as follows:

| | |
|---|---|
| Solution A: | |
| Anhydrous glucose | 15.0 g. |
| Distilled water | 250 ml. |
| Solution B: | |
| Ammonium succinate, or equivalent N | 0.98 g. |
| Distilled water | 250 ml. |

Solution C:

| | |
|---|---|
| $K_2HPO_4$ | 100.0 mg. |
| $KH_2PO_4$ | 700.0 mg. |
| $MgSO_4 \cdot 7H_2O$ | 201.0 mg. |
| $CaCl_2 \cdot 2H_2O$ | 5.95 mg. |
| $FeSO_4 \cdot 7H_2O$ | 5.00 mg. |
| $ZnSO_4 \cdot 7H_2O$ | 4.40 mg. |
| $MnSO_4$ | 2.75 mg. |
| NaCl | 2.55 mg. |
| $CuSO_4$ | 0.27 mg. |
| $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | 1.82 mg. |
| Distilled water | 480 ml. |

Solution D:

20 ml. of a stock solution giving the following concentrations per liter medium:

| | |
|---|---|
| *i*-Inositol | 1000 μg. |
| Thiamine HCl | 100 μg. |
| Pyridoxine HCl | 100 μg. |
| Calcium pantothenate | 100 μg. |
| Nicotinamide | 50 μg. |
| *p*-Aminobenzoic acid | 50 μg. |
| Biotin | 5 μg. |

The vitamin solutions were sterilized by passage through ultra-fine fritted glass filters. Solutions A, B, and C were autoclaved with an aluminum foil cover, which was replaced by a sterile cotton plug immediately after the autoclave was opened. Dry glassware was autoclaved with an aluminum foil cover placed over the cotton plug. After the solutions had been pooled, 50 ml. of the completed medium were dispensed in 250 ml. Erlenmeyer flasks. The final pH of the medium was 5.9. All glassware used was cleaned with chromic acid.

Preparation of the media used to determine carbon utilization was modified as follows: an amount of carbohydrate equivalent to a rate of 15 g. glucose/liter was dispensed per dry 250 ml. Erlenmeyer flask and after the addition of 1 ml. water was autoclaved. Fifty milliliters of sterile basal medium was then added to each flask.

Appropriate concentrations of filter-sterilized solutions of vitamins and compounds suspected of having vitamin-like properties were added to 50 ml. sterile basal medium in the study of growth factor requirements. Oleic acid, pimelic acid, and aspartic acid were neutralized before filtration. The final concentration of ethanol in media containing D-homobiotin, D-norbiotin, biocytin, and biotin L-sulphoxide did not exceed 0.03%. Ethanol controls revealed that this concentration was not inhibitory to growth. Yeast extract ash (600°/12 hours) in amounts equivalent to a rate of 180 mg./liter medium was autoclaved in 1 ml. water in 250 ml. Erlenmeyer flasks. Fifty milliliters of sterile medium was then added.

*Measurement of Growth*

Growth was measured as the dry weight of mycelium per flask culture and recorded as the mean of three or five replicas and its standard error. The mycelium of each culture was collected on heat-dried, individually tared Whatman No. 2 filter papers and weighed after drying 48 hours at 85° C. followed by at least 4 hours at room temperature in a desiccator. The pH of each culture was determined after filtration.

From a preliminary study it was found that results obtained from repetitions of an experiment varied significantly (Table I), and for this reason only data obtained from simultaneously-conducted experiments were considered for statistical comparison. The data of this table also reveal that replicas can be homogeneous although, as can be seen from some of the standard errors in various tables, they were not always so. This lack of uniformity in the internal variation was attributed to the use of small inocula (Fig. 1). The amount of inoculum used was normally of the order of 0.2–0.4 ml. For the purpose of this study, however, eliminating the carry-over of nutrients by the use of small inocula was considered of sufficient importance to outweigh the attendant disadvantages.

The significance of difference between growth responses was determined either by the $t$ test or through use of the analysis of variance. When using the latter, the means were compared by the variance ratio. Significantly different means were isolated from the analysis of variance using the total fiducial limit

TABLE I

EVIDENCE THAT UNCONTROLLED VARIATION BETWEEN EXPERIMENTS IS SIGNIFICANT BUT THAT REPLICATIONS WITHIN AN EXPERIMENT ARE HOMOGENEOUS

(mg. mycelium/flask)

| | PRL 1557, 10 days' incubation | | | | | |
|---|---|---|---|---|---|---|
| | Carbon source as variable | | | | | |
| | Glucose | | Sucrose | | Cellobiose | |
| Replica | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 |
| 1 | 310.5 | 350.6 | 337.7 | 390.3 | 292.2 | 352.8 |
| 2 | 317.3 | 347.6 | 344.1 | 373.9 | 306.3 | 355.1 |
| 3 | 314.3 | 341.9 | 366.5 | 384.9 | 286.7 | 342.4 |
| Mean | 314.0 | 346.7 | 349.3 | 383.0 | 295.1 | 350.1 |
| Variation within replicas | | | | | | |
| $Chi^2$* | 0.07 | 0.11 | 1.30 | 0.35 | 0.69 | 0.26 |
| (2 d.f.) | $P > 0.95$ | $P > 0.90$ | $P > 0.50$ | $P > 0.80$ | $P > 0.70$ | $P > 0.80$ |
| Variation between experiments | | | | | | |
| $t$ | 6.0 | | 2.9 | | 6.9 | |
| (4 d.f.) | $P < .01$ | | $P < .05$ | | $P < .001$ | |

NOTE: Experiments 1 and 2 differ by being initiated 48 hr. apart and by having different inocula. Flasks of Experiment 1 were inoculated with an equivalent of 0.20 mg. mycelium dry weight, and those of Experiment 2, 0.24 mg. mycelium dry weight.

*Using the mean as the expected frequency.

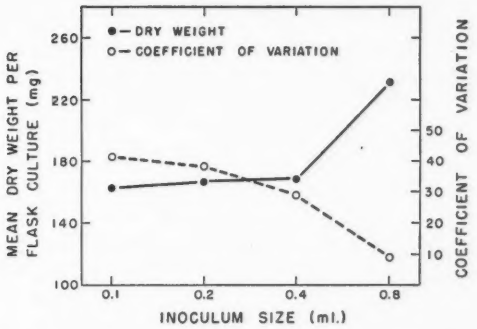


FIG. 1. The effect of inoculum size on total growth (10 days) and on variation within replicas of five (0.1 ml. inoculum is equivalent to 0.1 mg. dry weight of mycelium).

obtained from the interaction error (Snedecor, page 221 (23)). The comprehensive method of Tukey (26) gave results identical with the method of comparing individual means from an analysis of variance using the fiducial limit. The fiducial limit method was considered satisfactory for the purpose of this study.

*Growth Conditions*

An incubation period of 10 days was selected when it was found that no increase in dry weight of cultures grown on a medium containing glucose, ammonium succinate, vitamins, and minerals occurred after an 8–10 day interval. Unless otherwise indicated, cultures were grown for 10 days on a Gump shaker rotating at approximately 225 r.p.m. with a radius of motion of 1 inch. The temperature ranged from 26° to 28° C. The culture room was exposed to diffuse natural light from the northeast.

The requirement for carbon dioxide was tested by growing agar plate cultures in a chamber supplied with $CO_2$-free air. Conidiospores obtained from agar slant cultures were used to inoculate a semisolid medium consisting of the medium described earlier modified by the addition of 1.5% agar which had been washed with hot ethanol, water, and then freeze-dried. These plate cultures, with covers removed, were placed in a glass chamber and supplied with carbon-dioxide-free air at a rate of 460 ml. per minute.

## Results

*Utilization of Nitrogen*

Seven nitrogen compounds were tested (Table II). Only limited growth occurred when nitrogen was supplied as an inorganic salt. Growth on media containing ammonium salts was accompanied by a decrease in pH. Nitrate nitrogen was poorly utilized. Ammonium succinate was selected as the nitrogen source to be used in the remainder of this investigation because of its significance in the alkaloid production studies of Abe and co-workers (1).

TABLE II

GROWTH ON VARIOUS NITROGEN SOURCES

(Unless otherwise indicated, the data of these tables refer to cultures grown 10 days at 27° C. on a Gump shaker)

| Nitrogen source | PRL 1555 Mg. mycelium/flask | PRL 1555 pH | PRL 1556 Mg. mycelium/flask | PRL 1556 pH | PRL 1557 Mg. mycelium/flask | PRL 1557 pH |
|---|---|---|---|---|---|---|
| $(NH_4)_2SO_4$ | 50.8 ± 1.3* | 3.0 | 43.0 ± 2.9 | 3.2 | 25.3 ± 0.9 | 2.1 |
| $(NH_4)_2HPO_4$ | 142.4 ± 11.8 | 3.6 | 11.5 ± 1.3 | 3.6 | 15.8 ± 0.9 | 3.6 |
| $KNO_3$ | 24.8 ± 3.7 | 6.7 | 22.8 ± 4.9 | 6.5 | 81.6 ± 3.7 | 6.6 |
| Glutamate | 338.5 ± 26.4 | 8.4 | 296.2 ± 35.5 | 8.4 | 271.0 ± 18.7 | 8.4 |
| Asparagine | 280.6 ± 28.2 | 5.1 | 295.6 ± 38.2 | 5.7 | 254.5 ± 17.6 | 5.5 |
| Ammonium succinate | 315.8 ± 16.8 | 5.3 | 131.9 ± 23.1 | 5.5 | 227.2 ± 26.9 | 4.6 |
| Urea | 263.5 ± 20.5 | 7.4 | 170.9 ± 39.3 | 6.8 | 137.6 ± 14.7 | 5.2 |

*Standard error.

Inoculum: 1555 equivalent to 0.54 mg. dry mycelium per flask.
1556 equivalent to 0.36 mg. dry mycelium per flask.
1557 equivalent to 0.44 mg. dry mycelium per flask.

TABLE III

GROWTH ON VARIOUS CARBON SOURCES

| Carbon source | PRL 1555 Mg. mycelium/flask | PRL 1555 pH | PRL 1556 Mg. mycelium/flask | PRL 1556 pH | PRL 1557 Mg. mycelium/flask | PRL 1557 pH |
|---|---|---|---|---|---|---|
| None | 2.0 ± 0.1 | 5.9 | 1.9 ± 0.1 | 5.8 | 2.2 ± 0.1 | 5.9 |
| Glucose | 283.4 ± 4.1 | 4.8 | 158.8 ± 3.8 | 5.2 | 228.3 ± 3.0 | 6.2 |
| Fructose | 268.4 ± 5.9 | 5.9 | 216.1 ± 2.5* | 6.4 | 231.0 ± 18.5 | 5.1 |
| Mannose | 246.4 ± 6.8 | 4.8 | 124.4 ± 2.9 | 4.7 | 185.0 ± 21.6 | 6.4 |
| Mannitol | 22.3 ± 5.9 | 8.0 | 32.5 ± 1.1 | 8.2 | 37.7 ± 1.8 | 8.1 |
| Xylose | 6.7 ± 0.1 | 6.5 | 1.9 ± 0.4 | 6.7 | 1.6 ± 0.5 | 6.7 |
| Ribose | 28.3 ± 2.4 | 5.9 | 8.6 ± 0.4 | 8.3 | 4.3 ± 0.7 | 7.3 |
| Sucrose | 251.9 ± 7.5 | 5.9 | 132.4 ± 6.7 | 5.1 | 198.1 ± 9.9 | 6.7 |
| Maltose | 9.6 ± 0.2 | 8.6 | 12.1 ± 0.4 | 8.8 | 11.0 ± 0.2 | 8.7 |
| Cellobiose | 288.8 ± 16.1 | 5.2 | 164.1 ± 3.6 | 5.1 | 238.2 ± 9.4 | 5.7 |
| Starch | Trace | 8.2 | Trace | 7.1 | Trace | 7.4 |
| Powdered cellulose | Trace | 8.1 | Trace | 7.3 | Trace | 7.4 |
| Carboxy methyl cellulose | Trace | 7.5 | Trace | 7.9 | Trace | 7.7 |
| Methyl cellulose | Trace | 7.5 | Trace | 7.9 | Trace | 7.7 |

*Separate experiment.

Inoculum: 1555 equivalent to 0.35 mg. dry mycelium per flask.
1556 equivalent to 0.22 mg. dry mycelium per flask.
1557 equivalent to 0.31 mg. dry mycelium per flask.

*Utilization of Carbohydrates*

The three strains behaved similarly (Table III). They grew best on glucose, fructose, and cellobiose; significantly less on sucrose and mannose; and very poorly on the remaining compounds. The treatment of cellulose affects its physical properties, which in turn affects its availability (20, 21), and three forms of cellulose were therefore tested but none was utilized.

Growth was relatively poor on mannitol and maltose but the published reports of growth are not in agreement (16, 18, 27). The behavior of these three strains was therefore rechecked by a simultaneous comparison and again the cultures responded similarly.

*Carbon Dioxide*

An exogenous supply of carbon dioxide for initiation of growth by microorganisms has been reported by many investigators. These strains of *Claviceps purpurea* were examined for this requirement. Growth did not occur in $CO_2$-free air by 11 days whereas abundant growth was observed in the controls.

*Vitamin Requirements*

The three strains did not grow in the synthetic medium previously described when the vitamin solution (solution D) was omitted. Preparation of media in which each of the vitamins was in turn omitted from solution D showed that the three strains required biotin (Table IV) although a biotin requirement has not previously been reported for *Claviceps purpurea* (18, 22). The apparent requirement of PRL 1556 for nicotinamide was not substantiated by further tests, and the apparent reduced growth of PRL 1557 in the absence of thiamine was concluded not to be real (Table V).

The biotin concentration of the medium beyond which no further increase in growth occurred was less than 0.6 μg. per liter (Table VI). The optimal level would be expected to vary with the inoculum since some biotin contamination of vegetative inocula is unavoidable; hence an exact determination of the optimal level was not made.

Desthiobiotin (17), biocytin (31), biotin L-sulphoxide (30), homobiotin (6), and norbiotin (6) have been shown to replace biotin for certain microorganisms. In addition, desthiobiotin, homobiotin, and norbiotin have been shown to inhibit the growth of some fungi and bacteria in media containing biotin (6, 10, 14). These compounds were tested for their ability to replace, on an equimolar basis, an exogenous supply of biotin for these strains (Table VII). Biotin L-sulphoxide and biocytin partially replaced biotin. D-Desthiobiotin

TABLE IV

THE EFFECT OF VITAMINS ON GROWTH

| Vitamin omitted | PRL 1555 Mg. mycelium /flask | pH | Significance | PRL 1556 Mg. mycelium | pH | Significance | PRL 1557* Mg. mycelium | pH | Significance |
|---|---|---|---|---|---|---|---|---|---|
| None | 242.3 ± 12.6 | 7.4 | NS | 144.7 ± 1.5 | 4.6 | NS | 210.1 ± 24.9 | 4.3 | NS |
| All | 6.1 ± 0.1 | 6.1 | S | 0.2 ± 0.1 | 6.5 | S | 3.9 ± 0.1 | 6.3 | S |
| Biotin | 6.7 ± 0.2 | 6.1 | S | 2.5 ± 0.1 | 6.6 | S | 10.5 ± 0.3 | 6.3 | S |
| Thiamine | 214.1 ± 8.2 | 7.8 | NS | 146.3 ± 3.9 | 5.1 | NS | 179.2 ± 29.6 | 4.4 | NS |
| Pyridoxine | 265.3 ± 13.2 | 5.2 | NS | 137.7 ± 4.0 | 5.5 | NS | 209.9 ± 7.7 | 4.3 | NS |
| Nicotinamide | 217.3 ± 17.0 | 4.9 | NS | 96.1 ± 20.7 | 4.3 | S | 214.2 ± 10.4 | 4.3 | NS |
| Pantothenate | 242.7 ± 19.9 | 7.6 | NS | 140.0 ± 14.5 | 4.2 | NS | 227.7 ± 24.4 | 4.4 | NS |
| *p*-Amino benzoic acid | 261.4 ± 34.8 | 5.1 | NS | 107.9 ± 21.8 | 5.2 | NS | 217.9 ± 23.3 | 4.3 | NS |
| *i*-Inositol | 261.9 ± 13.5 | 6.3 | NS | 140.7 ± 3.4 | 5.2 | NS | 240.6 ± 19.7 | 4.2 | NS |

*Replica of five.

"NS" means not significant; "S" means significant ($P < .05$).

Inoculum: PRL 1555 equivalent to 0.35 mg. dry mycelium per flask.
PRL 1556 equivalent to 0.25 mg. dry mycelium per flask.
PRL 1557 equivalent to 0.33 mg. dry mycelium per flask.

TABLE V

A COMPARISON OF GROWTH OF PRL 1557 IN THE PRESENCE OF SEVEN B VITAMINS AND IN THE ABSENCE OF EACH OF THE SIX NON-ESSENTIAL VITAMINS

(mg. mycelium/flask)

| Replica flask | Vitamin omitted | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | Thiamine | Pyridoxine | Paba | Nicotinamide | Inositol | Pantothenate | Total |
| 1 | 199.1 | 207.7 | 210.0 | 248.3 | 221.5 | 224.7 | 293.6 | 1604.9 |
| 2 | 151.5 | 120.6 | 214.3 | 292.8 | 233.8 | 202.5 | 159.3 | 1374.8 |
| 3 | 224.3 | 136.7 | 189.9 | 193.7 | 227.5 | 279.2 | 191.0 | 1442.3 |
| 4 | 178.8 | 141.1 | 199.7 | 162.4 | 174.9 | 295.2 | 231.0 | 1383.1 |
| 5 | 297.1 | 289.7 | 235.5 | 192.5 | 213.5 | 201.4 | 263.7 | 1693.4 |
| Total | 1050.8 | 895.8 | 1049.4 | 1089.7 | 1071.2 | 1203.0 | 1138.6 | |

Analysis of variance

| Variation | | Degrees freedom | Sum of squares | Mean square | *F* |
|---|---|---|---|---|---|
| Total | | 34 | 76,372.8 | 2,246.2 | |
| Vitamins | | 6 | 10,782.6 | 1,797.1 | 1.24* |
| Thiamine | 1 | | 7,267.7 | 7,267.7 | 3.2* |
| Remaining six | 5 | | 3,651.5 | 730.3 | 3.5* |
| Replica | | 4 | 12,182.5 | 3,045.6 | 1.37* |
| Interaction | | 24 | 53,407.7 | 2,225.2 | |

*Not significant ($P > .05$).

was slightly active. Homobiotin and norbiotin were inactive. In a repetition of this experiment PRL 1555 grew 39% as well on biotin L-sulphoxide as on biotin and 19% as well on biocytin, PRL 1556 87% and 85%, PRL 1557 65% and 105% as well, respectively. Desthiobiotin was but slightly active and homobiotin and norbiotin not at all. Homobiotin and norbiotin were antibiotin factors for PRL 1555 but not for the other two cultures (Table VIII). These results, however, may have been influenced by variations in the biotin contamination of the three inocula.

Certain substances have been found to exert a sparing effect on, or to replace, the biotin requirement for certain bacteria. These are oleic acid (8, 12), aspartic acid (7), pimelic acid (28), aspartic acid plus oleic acid or some other long chain fatty acids (12), oleic acid plus Tween 40 (25), and Tween 80 (29). Apparently only aspartic acid exerts a sparing effect on the biotin requirement of some fungi (11, 13). The effect of their addition to the medium devoid of, or deficient in, biotin was determined for culture PRL 1555 because it possessed a long lag period even when given an excess of biotin. None of the compounds replaced biotin (Table IX). Both oleic acid and aspartic acid stimulated early growth but only oleic acid caused an increase in dry weight after 6 days' incubation (Table IX). These results were confirmed by a repetition of this experiment.

The effect of the remaining vitamins of solution D and of yeast extract ash (180 mg./liter) on this lag period was also examined. These supplements alone and in combination did not alter the lag period or total growth.

## TABLE VI

EFFECT OF THE INDICATED LEVEL OF BIOTIN ON GROWTH FOLLOWING A 10 DAY INCUBATION PERIOD*

| Biotin added, μg./liter | Culture number: PRL 1555 Mg. mycelium /flask | pH | Significance† | PRL 1556 Mg. mycelium /flask | pH | Significance† | PRL 1557 Mg. mycelium /flask | pH | Significance† |
|---|---|---|---|---|---|---|---|---|---|
| None | 2.6 ± 0.2 | 5.9 | S | 8.5 ± 3.9 | 5.7 | S | 26.3 ± 3.2 | 5.2 | S |
| 0.6 | 206.8 ± 14.5 | 4.5 | NS | 118.4 ± 5.8 | 4.3 | NS | 103.4 ± 11.6 | 3.9 | NS |
| 1.2 | 218.2 ± 7.5 | 4.5 | NS | 124.7 ± 4.9 | 4.2 | NS | 127.7 ± 12.7 | 3.9 | NS |
| 2.5 | 213.8 ± 2.9 | 4.5 | NS | 120.1 ± 2.6 | 4.2 | NS | 128.8 ± 11.4 | 4.1 | NS |
| 5.0 | 216.8 ± 8.0 | 4.5 | NS | 133.4 ± 1.8 | 4.3 | NS | 140.8 ± 8.2 | 4.3 | S‡ |
| 10.0 | 223.9 ± 8.2 | 4.5 | NS | 122.4 ± 0.6 | 4.1 | NS | 122.4 ± 3.9 | 3.8 | NS |

*1557 terminated at eight days because of commencement of growth in absence of biotin.
†"NS" means not significant; "S" means significant ($P < .05$).
‡Interpreted to be an artifact.
Inoculum: 1555 equivalent to 0.11 mg. dry mycelium per flask.
1556 equivalent to 0.14 mg. dry mycelium per flask.
1557 equivalent to 0.13 mg. dry mycelium per flask.

## TABLE VII

EFFECT OF BIOTIN, BIOTIN HOMOLOGUES, AND BIOTIN DERIVATIVES ON GROWTH $2.59 \times 10^{-9}$ *M**

| Supplement | PRL 1555 Mg. mycelium /flask | pH | PRL 1556 Mg. mycelium /flask | pH | PRL 1557 Mg. mycelium /flask | pH |
|---|---|---|---|---|---|---|
| None | 5.6 ± 1.1 | 5.8 | 6.9 ± 2.8 | 5.9 | 24.8 ± 12.5 | 5.2 |
| Biotin | 149.5 ± 10.2 | 4.0 | 163.9 ± 13.9 | 4.2 | 114.5 ± 3.9 | 3.9 |
| Biotin L-sulphoxide | 112.5 ± 5.7 | 4.2 | 109.9 ± 5.6 | 5.0 | 99.7 ± 1.2 | 3.7 |
| Biocytin | 69.8 ± 16.2 | 4.3 | 114.9 ± 18.5 | 4.0 | 107.5 ± 7.5 | 3.7 |
| Desthiobiotin† | 40.1 ± 12.2 | 5.1 | 33.9 ± 9.2 | 5.5 | 24.5 ± 2.1 | 4.8 |
| Homobiotin | 3.5 ± 1.0 | 5.8 | 3.0 ± 0.5 | 6.1 | 20.1 ± 4.5 | 5.0 |
| Norbiotin | 5.3 ± 0.9 | 5.8 | 3.9 ± 0.6 | 5.9 | 41.5 ± 10.5 | 4.9 |

*Suboptimal concentration of biotin.
†Calculated as D-desthiobiotin.
Inoculum: 1555 equivalent to 0.11 mg. dry mycelium per flask.
1556 equivalent to 0.16 mg. dry mycelium per flask.
1557 equivalent to 0.15 mg. dry mycelium per flask.

## TABLE VIII

EFFECT OF HOMOBIOTIN AND NORBIOTIN ON BIOTIN UTILIZATION*

| Supplement | Culture number: PRL 1555 Mg. mycelium /flask | pH | PRL 1556 Mg. mycelium /flask | pH | PRL 1557 Mg. mycelium /flask | pH |
|---|---|---|---|---|---|---|
| None | 1.9 ± 0.6 | 6.0 | 18.8 ± 3.4 | 5.4 | 18.1 ± 2.1 | 5.3 |
| Biotin | 126.4 ± 3.0 | 4.5 | 149.8 ± 0.4 | 4.4 | 109.2 ± 3.2 | 3.6 |
| Biotin + homobiotin | 58.8 ± 5.3 | 4.6 | 123.7 ± 12.5 | 3.8 | 119.2 ± 16.5 | 3.6 |
| Biotin + norbiotin | 87.9 ± 26.1 | 4.4 | 153.5 ± 8.9 | 4.4 | 126.6 ± 14.2 | 3.8 |

*Each supplement added to a concentration of $2.59 \times 10^{-9}$ *M*.
Inoculum: PRL 1555 equivalent to 0.93 mg. dry mycelium per flask.
PRL 1556 equivalent to 1.06 mg. dry mycelium per flask.
PRL 1557 equivalent to 0.94 mg. dry mycelium per flask.

TABLE IX

EFFECTIVENESS OF THE INDICATED SUPPLEMENTS IN REPLACING OR SPARING THE BIOTIN REQUIREMENT

Six day incubation

| Supplement | PRL 1555 | |
|---|---|---|
| | Mg. mycelium/flask | pH |
| None | 5.2 ± 0.6 | 5.4 |
| Biotin | 60.9 ± 8.0 | 4.4 |
| Biotin + oleate | 183.1 ± 8.9 | 3.7 |
| Biotin + aspartate | 41.1 ± 4.4 | 5.1 |
| Biotin + pimelate | 38.4 ± 4.9 | 5.1 |
| Biotin + Tween 80 | 65.2 ± 17.5 | 4.5 |
| Oleate | 10.6 ± 1.2 | 5.5 |
| Aspartate | 31.9 ± 6.1 | 5.0 |
| Pimelate | 14.3 ± 2.4 | 5.4 |
| Tween 80 | 8.1 ± 2.6 | 5.5 |
| Tween 80 + aspartate | 14.1 ± 1.8 | 5.6 |
| Tween 80 + oleate | 9.4 ± 4.0 | 5.5 |

NOTE: The biotin concentration (suboptimal) was $2.59 \times 10^{-9}$ *M*; oleate and Tween 80, 80 mg./liter each; and pimelate and aspartate, 100 mg./liter each.

Inoculum: equivalent to 0.27 mg. dry mycelium per flask.

## Discussion

The data reported in this paper provide a basis for successfully compounding various synthetic media for use in growing *Claviceps purpurea* for the purpose of studying ergot alkaloid production. Abe and co-workers (1) stress the fact that slow growth is required for alkaloid production. These data also provide a means of devising synthetic media allowing but a poor growth rate. Biotin was found to be required when using a synthetic medium and thoroughly washed inocula. With the exception of Robbins and Ma (22), previous investigators used either complex organic media, constituents capable of containing biotin (e.g. Mandels (15) found sucrose but not glucose to contain biotin), incompletely washed inocula, or various combinations of these conditions. Consequently, although it cannot be said that their cultures required an exogenous supply of biotin, it can be assumed the conditions employed by them probably would not demonstrate a biotin requirement should it exist.

The nutritional data of the various investigators differ in the main on comparative carbohydrate utilization. The three strains studied in this laboratory under identical conditions responded similarly to the carbohydrates examined.* This suggests the possibility that previously published differences might be due in part to the use of different basal media and inocula of differing histories and amounts.

*Recently a fourth culture of *Claviceps purpurea* (obtained from Dr. A. W. Henry, Department of Plant Science, University of Alberta) was found also to require biotin and to have the above described carbohydrate utilization pattern.

No utilization of inorganic ammonium salts has been reported and utilization by our three strains was limited, which may be a result of a drop in pH (5). Nitrate nitrogen utilization which was not accompanied by a drop in pH was poor, and this agrees with the published reports (18, 27).

It was concluded that biotin L-sulphoxide and biocytin can nearly, if not completely, replace an exogenous supply of biotin but that desthiobiotin only partially replaces biotin. Homobiotin and norbiotin were not effective in replacing biotin and they were antibiotin factors for one strain. The fact that the two cultures which were not inhibited contained contaminating biotin (as judged by the amount of growth in the absence of added biotin) may explain these differences in response. On the basis of examination of one strain, aspartic acid and oleic acid accelerate the onset of growth and oleate is sufficiently active to cause greater growth by 6 days.

While an optimal mineral basal medium was not developed, the fact that neither growth rate nor total growth was stimulated by the addition of yeast extract ash to the medium suggests a serious imbalance was not present.

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# LYSOZYME IN THE BACTERIOLYSIS OF GRAM-NEGATIVE BACTERIA

## I. MORPHOLOGICAL CHANGES DURING USE OF NAKAMURA'S TECHNIQUE[1]

E. A. GRULA[2] AND S. E. HARTSELL


## Abstract

Whole cells or the cell walls of 22 species representing 11 genera of Gram-negative bacteria were exposed to lysozyme using a modified Nakamura technique. The cell walls of all organisms contain the lysozyme substrate in differing amounts (two *Brucella* spp., *Proteus vulgaris* X-19, and *Vibrio cholerae* Chicago are possible exceptions) when evaluated spectrophotometrically and with the electron microscope. Using the latter technique, the sequence of events during bacteriolysis with lysozyme was observed. After exposure to lysozyme in saline, with the modified Nakamura technique and the phase microscope, cells were observed to either swell or shrink depending on the pH of the menstruum. This phenomenon apparently involves reversible hydration of cell proteins with concomitant changes in light transmission.


## Introduction

Since the discovery of lysozyme by Fleming in 1922, conflicting opinions have been expressed regarding the presence or absence of the lysozyme substrate in Gram-negative bacteria (3, 4, 8, 14, 15, 19, 20). In brief, it may be stated that opinions differed because of variation in: (1) source, purity, and preparation of lysozyme, (2) strain differences in organisms, and (3) the testing procedures used. Nakamura (12) demonstrated that suspensions of "lysozyme-resistant" bacteria could be cleared by alkali (pH 10.0) if the cells were first exposed to lysozyme in dilutions of hydrochloric acid. His testing procedure did not, however, receive studied application by most investigators.

Myerholtz and Hartsell (11) reported that the lysozyme substrate could be demonstrated in all Gram-negative bacteria assayed when the following test conditions were satisfied (modified Nakamura technique): pH 3.5 or lower during incubation of the cell–lysozyme mixture, temperature of 45° C. during incubation, incubation time of 1 hour, addition of sodium hydroxide to pH 10.0–10.5 after incubation before the temperature fell below 40° C., and cells not over 24 hours old.

Peterson and Hartsell (13) confirmed these results and reported that 135 species of 11 genera of Gram-negative bacteria showed differential clearing in alkali after incubation with lysozyme.

Although complete or partial clearing of a cell suspension by alkali after treatment with lysozyme at a low pH may be accepted as evidence for

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lysozyme action, it appeared that direct observation of the cells during the Nakamura treatment might shed more light on the entire phenomenon. Also, it appeared desirable to compare the lysis of Gram-negative bacteria to the lytic situation as observed with *Micrococcus lysodeikticus* (6, 7).

## Materials and Methods

Twenty-two species representing 11 genera of Gram-negative bacteria were studied using the following conditions supplemented with electron or phase microscopic observations. The genera included *Salmonella*, *Brucella*, *Klebsiella*, *Shigella*, *Neisseria*, *Pseudomonas*, *Escherichia*, *Proteus*, *Erwinia*, *Vibrio*, and *Pasteurella*.

All bacteria except the *Brucella* spp. (tryptose agar (2)) were grown for 18–21 hours on yeast water veal infusion agar at 25° C. (18). Before use, all cells were washed in two changes of double-distilled water. Also, all reagents were made with double-distilled water, unless otherwise specified. Reaction mixtures contained 2 ml. washed cells, 2 ml. lysozyme* (1 : 10,000), and 2 ml. of dilute hydrochloric acid to give a pH of 3.4–3.6. Control sets contained 2 ml. washed cells, 2 ml. distilled water, and 2 ml. of dilute hydrochloric acid. All mixtures were incubated at a temperature of 45° C. for 1 hour. Spectrophotometric readings were made at a setting of 540 m$\mu$ against a distilled water blank using a Coleman Universal Spectrophotometer, and samples for electron microscopy were taken at zero time and after 1 hour. After 1 hour, sodium hydroxide was added to the cell–lysozyme mixture to raise the pH to 10.0 or above. Immediately, readings and samples for microscopic study were again taken.

Since several investigators have demonstrated that the cell wall of sensitive bacteria contains the lysozyme substrate, cell wall preparations were obtained from the bacteria by the technique of Grula and Hartsell (7) and observed under their test conditions.

Techniques for electron microscopy and Alcian blue staining were the same as reported previously (6). All specimens were shadow-cast with chromium metal (whole cells at an angle of approximately 30 degrees; cell wall preparations at an angle of approximately 80 degrees). All preparations were air-dried and no fixation was attempted.

## Results

*Spectrophotometric Analysis*

The optical density obtained for each organism is given in Table I, wherein the organisms are arranged in order of decreasing sensitivity to lysozyme, on the basis of the percentage of total clearing due to lysozyme. These data also include the percentage of total clearing not due to lysozyme which occurred when alkali was added to the control mixtures. The relationship was inverse: reading the table from bottom to top (per cent change not due to lysozyme), a decreasing order for clearing in the presence of alkali is obtained.

*Crystalline egg-white lysozyme. Armour & Co., Chicago, Ill.

TABLE I

GRAM-NEGATIVE BACTERIOLYSIS

| Organism | Optical density | | | | |
|---|---|---|---|---|---|
| | Initial | Without lysozyme* | With lysozyme* | % change due to lysozyme | % change not due to lysozyme |
| *Pseudomonas aeruginosa* | .520 | .367 | .056 | 67.0 | 33.0 |
| *Neisseria catarrhalis* | .520 | .310 | .010 | 58.8 | 41.2 |
| *Escherichia coli* BM 12 | .520 | .329 | .076 | 56.9 | 43.1 |
| *Neisseria perflava* | .520 | .292 | .032 | 53.3 | 46.7 |
| *Pasteurella pseudotuberculosis* | .520 | .238 | .032 | 42.3 | 57.7 |
| *Escherichia coli* S-1 | .520 | .260 | .072 | 42.0 | 58.0 |
| *Salmonella cholerasuis* | .520 | .245 | .076 | 38.1 | 61.9 |
| *Klebsiella pneumoniae* BEPR | .520 | .278 | .143 | 35.8 | 64.2 |
| *Salmonella gallinarum* | .520 | .222 | .066 | 33.9 | 66.1 |
| *Klebsiella pneumoniae* 2M | .520 | .229 | .071 | 32.9 | 67.1 |
| *Pseudomonas syncyanea* | .520 | .199 | .041 | 32.9 | 67.1 |
| *Pasteurella multocida* | .520 | .188 | .066 | 26.7 | 73.3 |
| *Erwinia carotovora* Arkansas | .520 | .174 | .046 | 24.9 | 75.1 |
| *Shigella ceylonensis* | .520 | .260 | .168 | 23.3 | 76.7 |
| *Proteus mirabilis* | .520 | .215 | .137 | 20.4 | 79.6 |
| *Shigella dysenteriae* | .520 | .143 | .050 | 19.8 | 80.2 |
| *Erwinia carotovora* C-3 | .520 | .149 | .072 | 17.2 | 82.8 |
| *Vibrio cholerae* Ogawa | .520 | .149 | .076 | 16.5 | 83.5 |
| *Brucella suis* No. 5 | .520 | .410 | .390 | 15.4 | 84.6 |
| *Proteus vulgaris* X-19 | .520 | .245 | .208 | 11.9 | 88.1 |
| *Vibrio cholerae* Chicago | .520 | .076 | .066 | 2.2 | 97.8 |
| *Brucella suis* No. 9 | .520 | .358 | .358 | 0.0 | 100.0 |

*Followed by NaOH to pH 10.0 or above.

Although good clearing was obtained with *V. cholerae* Chicago, Table I shows that little or none of the clearing was due to lysozyme. Peterson and Hartsell (13) also reported the same observation. Studies with the electron microscope showed that the cell wall of this organism was destroyed in the presence of alkali without the prior need for lysozyme treatment. Destruction of the cell wall led to dispersion and hydration of the cellular contents, resulting in clearing of the suspension.

Throughout these studies, the bacterial suspensions always showed an increased viscosity after clearing in the presence of alkali. Although the amount of viscosity increase was not measured, the manifestation of such an increase argues for hydration of cellular proteins by the alkali (5) and possible leaching of nucleic acids.

### *Alcian Blue Staining*

Grula and Hartsell (6) demonstrated that the cell wall of the sensitive Gram-positive organism *Micrococcus lysodeikticus* stained very well with Alcian blue. This dye is specific for polysaccharide materials (9). Because Alcian blue was released during lysozyme digestion when the cell walls were not saturated and because cell walls saturated with the dye were not digested by lysozyme (7), whole cells and isolated cell walls of Gram-negative bacteria were stained with Alcian blue to see if a positive correlation existed regarding lysozyme sensitivity and good staining of the cell walls.

The cell walls of the Gram-negative bacteria did not stain well. Usually, the staining was light blue and even throughout, which is often characteristic of cytoplasmic staining. *N. perflava* and *K. pneumoniae* 2M were exceptions. Cell walls from *N. perflava* stained intensely. Cell wall staining was not as intense with *K. pneumoniae* 2M. *N. perflava* was fourth and *K. pneumoniae* was seventh in clearing with lysozyme (Table I). They were 19th and 16th, respectively, in clearing with alkali in the absence of lysozyme (Table I). Cell wall staining was decreased in both organisms after lysozyme digestion.

Because good cell wall staining of other organisms which had shown good response to lysozyme did not occur (*N. catarrhalis* and *P. aeruginosa*), it appears that this dye is not a reliable indicator for predicting the lysozyme sensitivity of all bacterial cells. This does not mean, however, that Alcian blue does not stain the substrate of lysozyme or essentially polysaccharide complexes in the cell wall.

Poor staining of the bacterial cell wall by Alcian blue in organisms very sensitive to lysozyme by Nakamura technique could occur because the lysozyme substrate need not be present in large amounts to obtain good lysis or the lysozyme substrate is covered or bound within the cell wall and not exposed for staining in cells which have not had some form of pretreatment. Regarding the latter possibility, staining by Alcian blue was increased appreciably with cells of *S. gallinarum* after pretreatment for 1 hour at 45° C. with hydrochloric acid (pH 3.5).

*Electron Microscopic Observations*

During treatment of the whole cells with the Nakamura technique, several degrees of change appeared to exist; however, all appeared to be variations in amount only, particularly the final reaction with alkali. To ensure clarity, the sequences will be reported stepwise.

(1) Cells incubated in the presence of acid (pH 3.5; no lysozyme) at 45° C. for 1 hour showed two effects: partial coagulation of the cells, which gave them a speckled appearance (Fig. 13), or coagulation of the entire cell, which made them rather electron-opaque (Fig. 2). Untreated cells did not show this coagulation (Figs. 1 and 12). Because the coagulation occurred only at the low pH either in the presence or absence of lysozyme (Figs. 2 and 3), it was due to the low pH (3.5) and to the temperature of incubation (45° C.). Further, because isolated cell walls treated in the same manner showed no coagulation (Figs. 7, 8, 11), the major portion of the reaction occurred within the cytoplasm of the cells. The reaction apparently involved protein denaturation and coagulation since these cells appeared "fixed", i.e., they appeared smaller than untreated cells and were more electron-opaque. Further, they did not flatten on the grids; instead, they retained a three-dimensional state as evidenced by the distinct shadows.

This coagulation and increased density within the cells was expressed by a drop in % transmission (T) which occurred to some extent with all bacteria screened after incubation for 1 hour at pH 3.5.

Also, it is to be noted that the isolated cell walls of *V. cholera* Ogawa possessed the same form as the intact cells. This observation is consistent with the viewpoint of Salton (16) and Mitchell and Moyle (10) regarding the rigidity of bacterial cell walls.

(2) When the cells were incubated in the presence of acid and lysozyme, no effects differing from those described above occurred with regard to cellular morphology and density changes. They did not swell and they did not burst, nor did they decrease in density as might have been expected. *N. perflava* was an exception since these cells showed a slight density decrease.

(3) When alkali was added to the cells incubated in acid as described under (1), the general effect was a decreased cell density (Fig. 4), with some swelling. With some organisms, e.g., *V. cholera* Chicago, dispersion of the entire cell occurred.

(4) When alkali was added to the cells incubated in acid with lysozyme as described under (2), the effects ranged from little or no decreased cell density through rather complete "ghost" formation (Figs. 14, 15) to cellular disruption and dispersion (Fig. 5). Ghost formation and/or cellular dispersion were the more common effects. Cells resistant to lysozyme under these conditions (*Brucella* spp.) showed little or no change.

Observations made when isolated cell walls were used were often in good agreement with those made with whole cells except that density changes were not apparent. Generally, dispersion of them was seen only after alkali was added to the tubes containing lysozyme (Figs. 6–10). However, disruption of the cell walls of some species by alkali occurred in the absence of lysozyme (*P. vulgaris* X-19 and *V. cholera* Chicago). *N. perflava* was also different since the cell walls from this organism were digested by lysozyme without the addition of alkali.

The general correlation that greater destruction of the cell wall brought about increased clearing of whole cells by alkali after lysozyme digestion demonstrates again that the cell walls of bacteria are the primary structures digested by lysozyme. Further, it is again observed that cell wall digestion by lysozyme aids alkali in clearing the bacterial suspensions (7).

*Observations with the Phase Microscope*

Studies with the phase microscope were initiated because continuous observation of live cells was not possible with the electron microscope. Also, the problem of cell swelling could not be satisfactorily resolved with the electron microscope because of the possibility that artifacts due to flattening could occur. A relatively sensitive organism (*S. gallinarum*) and an insensitive organism (*P. vulgaris* X-19) were first studied to observe what morphological changes occurred during the Nakamura treatment. Cells were treated with or without lysozyme at pH 3.5 at 45° C. in saline for 1 hour before addition of alkali. They were observed in wet mount preparations using a Spencer phase contrast microscope, with the bright high contrast oil immersion objective. The wet mount preparations were made by placing

two drops of the cell suspension from a tube incubated for 1 hour (45° C. or 25° C.) on a clean glass slide and covering them with a cover slip. Sodium hydroxide (two drops of saline having a pH of 10.5) was added with a capillary pipette at the side of the cover slip and allowed to diffuse into the unsealed system. Observations are reported separately for each organism.

*S. gallinarum*

(1) Normal cells appeared homogenous and of bright intensity throughout.

(2) Cells at pH 3.5 (no lysozyme) for 1 hour appeared more dense (still brighter); no other changes were observable. Addition of alkali caused the density of most of the cells to decrease somewhat and some swelling occurred.

(3) Cells at pH 3.5 with lysozyme for 1 hour also appeared more dense; no other changes were observable. Addition of alkali caused most of the cells to decrease noticeably in density. Also, they swelled very quickly (1 to 3 seconds) to two or three times normal size and showed a tendency to round out. The "ghosts" which remained appeared similar to leached ghosts of *S. gallinarum* seen in electron micrographs although some leached and rounded forms similar to those shown in Fig. 15 were also evident.

*P. vulgaris X-19*

(1) Normal cells appeared homogenous and of bright intensity throughout.

(2) Cells at pH 3.5 for 1 hour appeared slightly more dense; no other changes were observable. Addition of alkali caused a density decrease that was quite noticeable; concurrently, swelling in some cells was evident.

(3) Cells at pH 3.5 with lysozyme for 1 hour appeared to be somewhat more dense; no other changes were observable. Addition of alkali caused some of the cells to diminish in density and to swell. The density decrease was not as great as that which occurred with *S. gallinarum*. Also, most of the cells did not change other than to decrease slightly in density. *P. aeruginosa*, *E. carotovora*, and *E. coli* behaved similarly.

Because the swelling and density changes of the cells were so striking, the reaction was again studied using *P. aeruginosa* and *S. gallinarum*. Since these cells, when treated with lysozyme at pH 3.5 at 45° C. for 1 hour, always swelled and decreased in density after addition of alkali to pH 10.0, it appeared appropriate to see if the swelling and density decrease could be reversed by readjusting the pH to 3.5 with hydrochloric acid. The results, shown in Plate IV, obtained using *P. aeruginosa* demonstrate that the reaction was almost completely reversible. That is to say, a shrinking of swollen cells with a concomitant increase in density occurred when the pH was adjusted back to 3.5. It is to be noted, however, that this system did not allow for complete reversibility of the reaction as reported for bacterial cells in solutions containing varying amounts of organic or inorganic compounds (10). Although the reversibility of the reaction was very obvious and striking, it was more surprising to observe that cells which had been swollen and then shrunken could again be made to swell and decrease in density if the pH was again

adjusted to 10.0. The correlation of cell size and density changes with spectrophotometric readings and pH are given (Figs. 16–20).

Additional experiments using *P. aeruginosa* (18 hour) showed that the shrinking and swelling phenomenon could be reversed 10 times (pH ranges were between 3.0–3.5 at the lower limit and 9.5–10.0 at the upper limit). After 10 continuous exposures, the pH was raised to 11.6 with 2 *N* sodium hydroxide; at this value the cells were disrupted. Older cells of *P. aeruginosa* (96 hours) could also be made to shrink and swell. Induction of swelling and shrinking of *P. aeruginosa* using lysozyme and the same pH adjustments in a distilled water system was also possible, thus plasmoptic effects would appear not to be involved in this phenomenon.

Staining of the swollen bacteria in wet mount preparations at pH 10.0 (at which pH the proteins will carry a net negative charge) with crystal violet or methylene blue (chromatophore positively charged) was very good whereas staining with Alcian blue (primarily a polysaccharide stain) was negative. The reversibility of the shrinking and swelling reaction by adjustment either to acidic (pH 3.5) or basic (pH 10.0) conditions and the relative ease with which these swollen cells stained with basic stains would suggest that the swelling and shrinking phenomenon, with the accompanying density changes, involve chiefly cellular proteins.

Although swelling of Gram-negative bacteria did not occur during treatment with lysozyme at pH 3.5, these studies have demonstrated that subsequent adjustment of the pH to 10.0 will induce swelling during which time hydration and solubilization mechanisms are set in motion. Brown (1) stated that protein structures undergoing hydration swell. Although electron micrographs had shown that the cells were larger after alkali clearing, it always appeared possible that flattening of leached, and therefore flaccid, ghosts during drying could account for the apparent increase in size. In wet mount preparations, such an occurrence appears highly improbable.

## Discussion

Electron microscopic results (7) showed that digestion of the cell wall of *M. lysodeikticus* by lysozyme could occur at pH 3.5. However, the digestion was masked when the reaction (5% T over-all) was observed spectrophotometrically since only limited clearing could occur at the low pH. Following the same general pattern, no clearing of the Gram-negative bacteria occurred at pH 3.5 with lysozyme. Therefore, there is little difference between the amount of clearing obtained with Gram-negative bacteria or *M. lysodeikticus* if incubation with lysozyme is performed at pH 3.5. A great difference in the response of *M. lysodeikticus* and the Gram-negative bacteria to lysozyme can be shown with the spectrophotometer, however, if the cells are treated with lysozyme at pH 6.0 to 7.0. Under these conditions, *M. lysodeikticus* will clear from 30 to 80 or 90% T *without* addition of alkali whereas most Gram-negative bacteria (*N. perflava* and *P. aeruginosa* are exceptions) will clear very little even *after* addition of alkali. However, the amount of final clearing obtained

with the Gram-negative bacteria can be increased (although it varies among species) if the cells are incubated with lysozyme at pH 3.5 prior to the addition of alkali.

With the electron microscope, another difference is apparent. Cell walls of *M. lysodeikticus* are digested by lysozyme at pH 3.5. The cell walls of Gram-negative bacteria (*N. perflava* and *P. aeruginosa* are exceptions), however, are not visibly digested by lysozyme at the low pH. It is only after the addition of alkali that complete or partial disruption of the cell walls and whole cells occurs. The amount of disruption depends on the final pH and the cell walls used.

The reversible swelling and shrinking phenomenon undoubtedly involves hydration of proteins and can be interpreted in terms of Seifriz's theory of protoplasm structure (17) and the concept of a protein framework of Widra (21). Seifriz postulated that proteins in protoplasm exist as fibers bound together in an orderly three-dimensional lattice, or crystal. Because the fibers overlap in the non-hydrated state, continuity of the structure would be maintained during imbibition of water and consequent swelling. The maintenance of this lattice structure would permit reversion to the "normal" non-hydrated state.

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## PLATE I

FIGS. 1–5. Electron micrographs. Initially, intact cells were used. All magnifications approximately ×6500.

FIG. 1. *P. syncyanea.* Untreated.

FIG. 2. *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5.

FIG. 3. *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme.

FIG. 4. *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5. NaOH added.

FIG. 5. *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added.

## PLATE II

FIGS. 6–10. Electron micrographs. All magnifications approximately ×6500.

FIG. 6. Cell walls of *P. syncyanea.* Untreated.

FIG. 7. Cell walls of *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5.

FIG. 8. Cell walls of *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme.

FIG. 9. Cell walls of *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5. NaOH added.

FIG. 10. Cell walls of *P. syncyanea.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added.

## PLATE III

FIGS. 11–15. Electron micrographs. All magnifications approximately ×6500.

FIG. 11. Cell wall of *V. cholerae* Ogawa. Treatment: 1 hr. at 45° C. at pH 3.5.

FIG. 12. *P. pseudotuberculosis.* Untreated.

FIG. 13. *P. pseudotuberculosis.* Treatment: 1 hr. at 45° C. at pH 3.5.

FIG. 14. *P. pseudotuberculosis.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added.

FIG. 15. *K. pneumoniae* BEPR. Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added.

## PLATE IV

FIGS. 16–20. Phase contrast photomicrographs. All magnifications approximately ×4000.

FIG. 16. *P. aeruginosa.* Untreated. % T, 30.

FIG. 17. *P. aeruginosa.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. % T, 23.

FIG. 18. *P. aeruginosa.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added to pH 10.0. % T, 85.

FIG. 19. *P. aeruginosa.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added to pH 10.0 then HCl to lower pH back to 3.5 % T, 43.

FIG. 20. *P. aeruginosa.* Treatment: 1 hr. at 45° C. at pH 3.5 with lysozyme. NaOH added to pH 10.0 then HCl to lower pH to 3.5, then NaOH again to pH 10.0. % T, 85.

Note (Figs. 18 and 20) the differences in density (hydration) between the inside and the periphery of the cells.

NOTE: Figs. 1–20 follow.

PLATE I

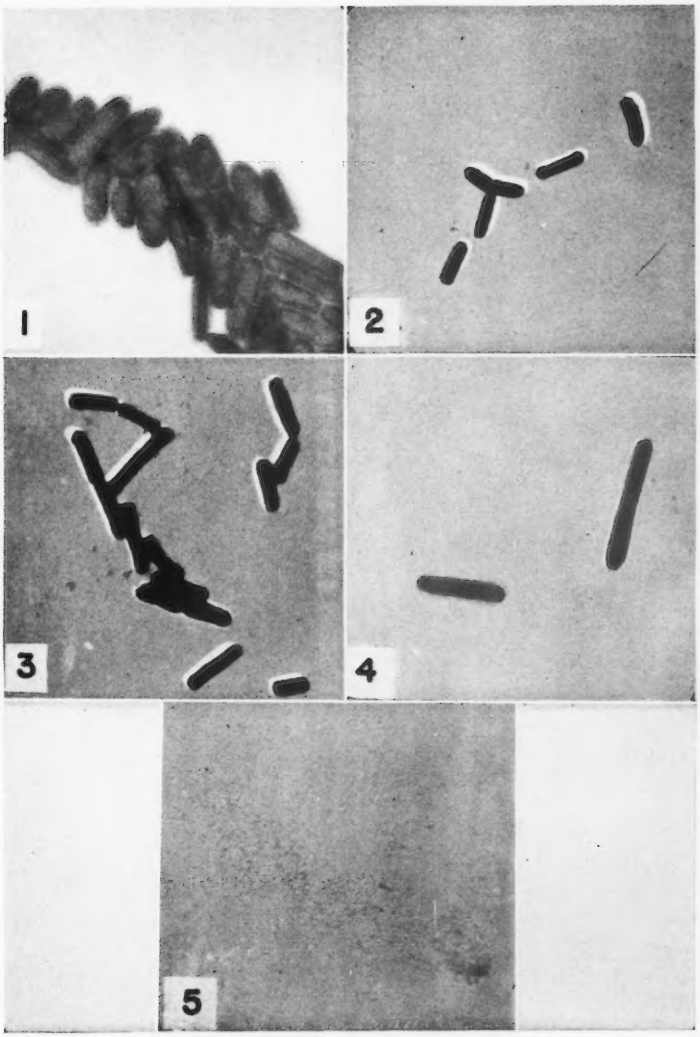

PLATE II

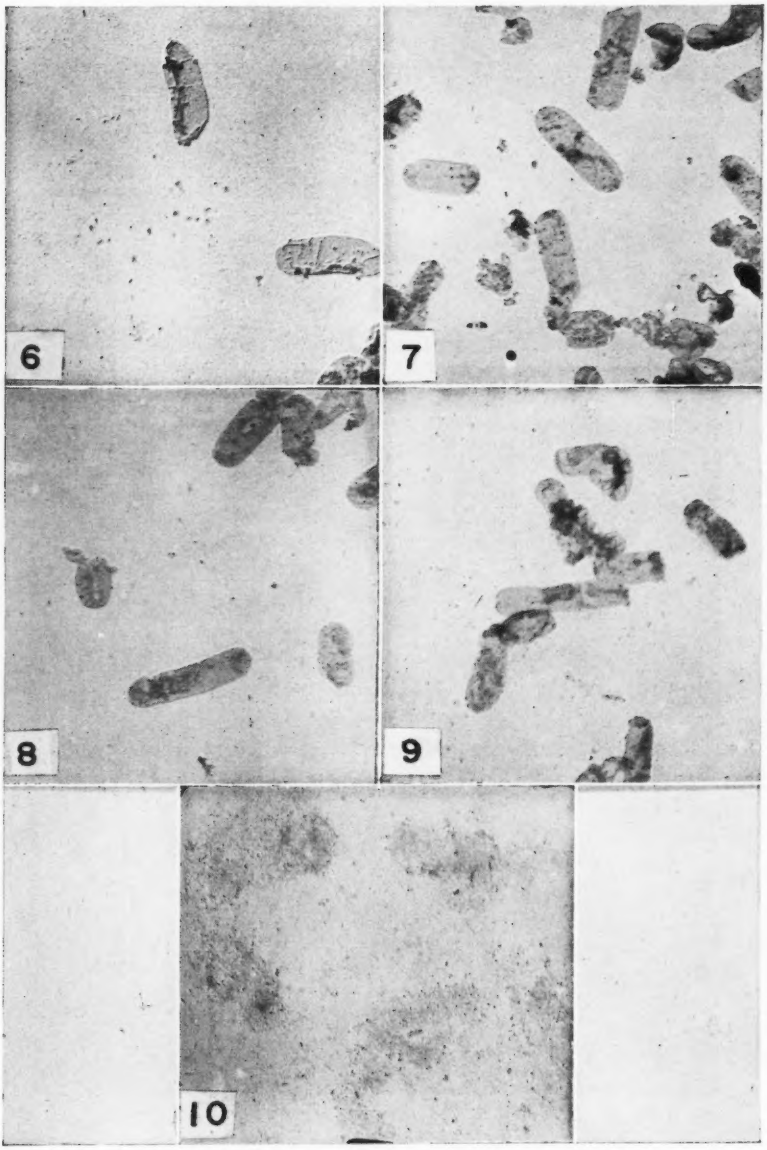


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PLATE III

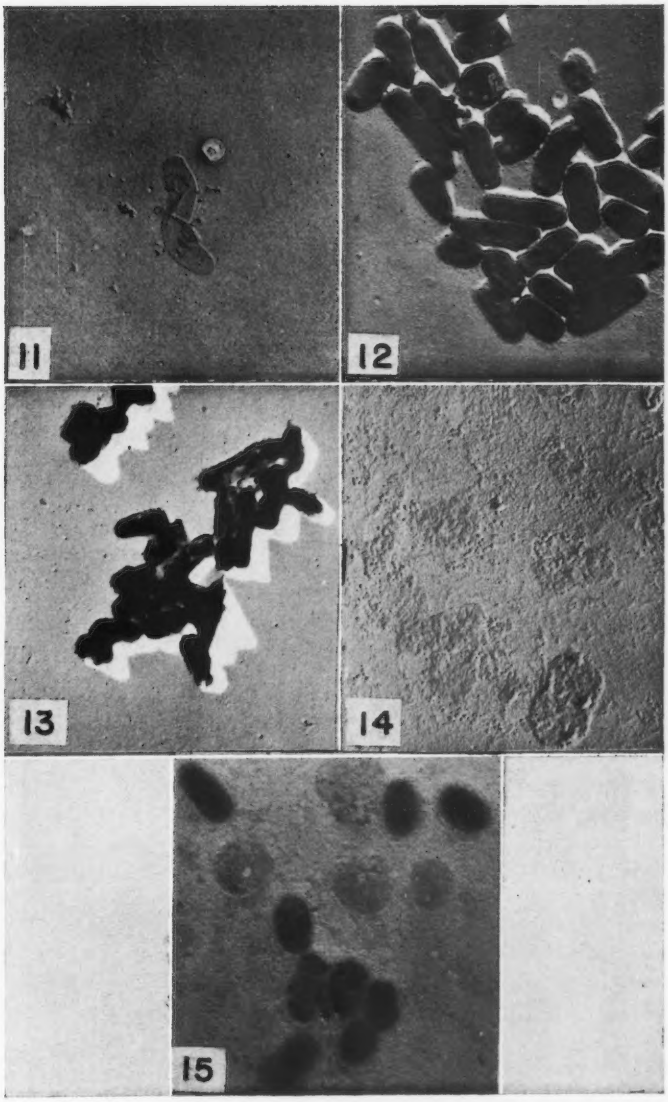


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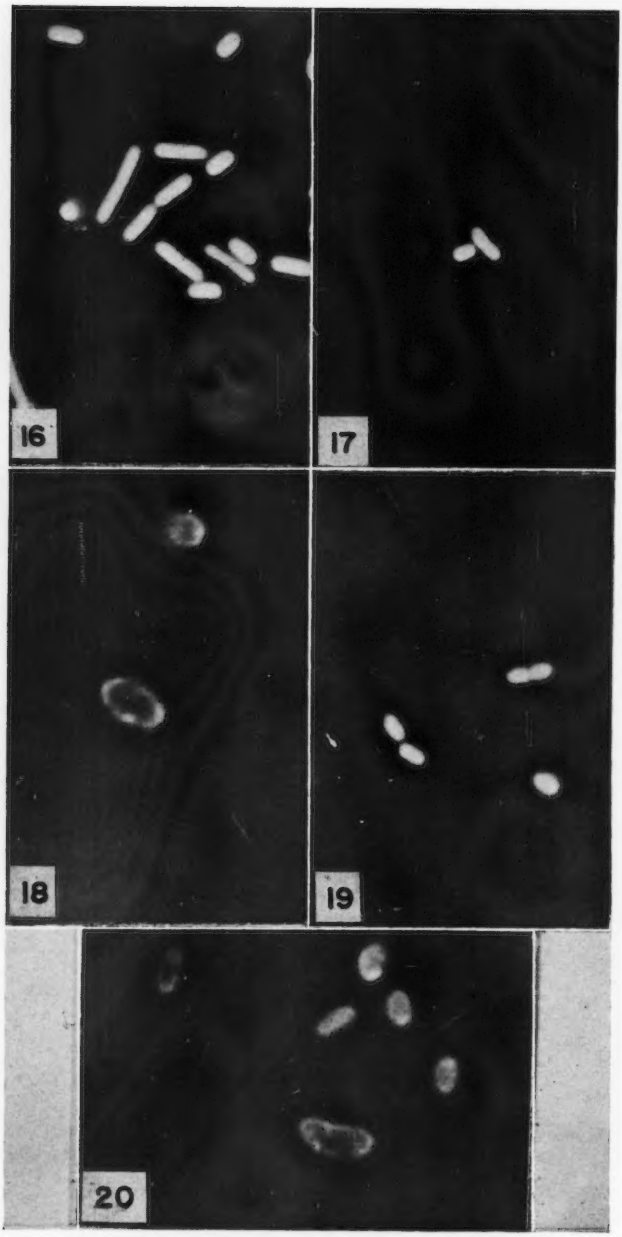
PLATE IV

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# LYSOZYME IN THE BACTERIOLYSIS OF GRAM-NEGATIVE BACTERIA

## II. FACTORS INFLUENCING CLEARING DURING THE NAKAMURA TREATMENT[1]

E. A. GRULA[2] AND S. E. HARTSELL


## Abstract

Nakamura conditions are helpful in demonstrating the presence of the lysozyme substrate in Gram-negative bacteria because: (1) the low pH (3.5) pretreatment uncovers or releases the substrate from a "bound" state within the cell wall for lysozyme digestion; (2) the addition of alkali (to pH 10.0 or above) allows for increased hydration and dispersion of the cell proteins once the lysozyme substrate has been removed. Increased hydration and dispersion alter the index of refraction of bacterial suspensions so that it is near the index of water, and clearing of the suspensions results. Irrespective of the acids and/or alkalies used, little difference was noted in their effect on clearing. The need for caution in attributing lack of clearing in bacterial suspensions to inactivation or inhibition of autolytic enzyme systems is discussed.


## Introduction

When testing for the presence of the lysozyme substrate in bacteria, the pH of the menstruum usually is between 6.2 and 6.8 since it is well established that best clearing occurs near neutrality. Nakamura (11) demonstrated that suspensions of "lysozyme resistant" bacteria (no clearing at neutrality) would clear in the presence of alkali (pH 10.0) if incubation with lysozyme was performed in various dilutions of hydrochloric acid.

Explanations for the use of the Nakamura technique in tests for the presence of the lysozyme substrate in Gram-negative bacteria are, however, lacking. Grula and Hartsell (8) using *Micrococcus lysodeikticus* observed that the clearing of lysozyme treated cells in the presence of alkali was based on prior digestion of the bacterial cell wall by lysozyme (partial or complete) which permitted intimate contact between alkali and the cytoplasm of the cell. Although they observed that cellular suspensions did not clear at pH 3.5, it was demonstrated with the electron microscope that lysozyme digestion of the cell wall did occur.

In an extension of this work (9), they demonstrated that although acid treatment was necessary to show that lysozyme had acted, the cell walls of typical Gram-negative bacteria were not visibly affected by lysozyme at pH 3.5. Only when alkali was added did a reaction occur. Because the response to lysozyme of typical Gram-negative bacteria is different from that of *M. lysodeikticus* and because most Gram-negative bacteria manifest a

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Contribution from the Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A. This paper is part of the thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology, Purdue University. Acknowledgment is expressed to the Eli Lilly Company, Indianapolis, Indiana, for the fellowship which made a portion of this work possible.

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good reaction to lysozyme only with the Nakamura technique, this report will attempt to explain why the rather extreme acid and alkaline pH adjustments used in the Nakamura treatment are helpful in demonstrating the presence of the lysozyme substrate in Gram-negative bacteria.

## Materials and Methods

Generally, the same procedures were used in this study as reported previously (7, 8, 9). Saline was substituted for distilled water to minimize the effects of plasmoptysis on the cells during repeated washings.

Cell-free systems for the study of autolytic enzyme activity were prepared from washed cells of *Pseudomonas aeruginosa* or *Erwinia carotovora* (18–21 hours) by three methods: (1) grinding of heavy cell suspensions with alumina for 15 minutes at 4° C., (2) three to nine alternate freezes (−9° C.) and thaws (25° C.) in distilled water or saline, or (3) sonic oscillation. During oscillation, the cells were agitated in distilled water or saline at maximum frequency for intervals of 10, 20, 30, or 60 minutes on a Raytheon Magnetostriction Oscillator, Model S-102A with a maximum frequency output of 9 kc.

Several modifications in the freeze–thaw technique were employed, namely: (1) single, double, or triple extractions using saline or distilled water, (2) extraction using 10 pH values from 1.9 to 9.5, (3) alcohol extraction at 1, 5, 10, 20, or 30% concentration, (4) addition of lysozyme during freezing and thawing, or (5) addition of cysteine during or after freezing and thawing.

The insoluble cell materials were always spun down at 5000 r.p.m. for 30 minutes at 4° C. on a small Servall angle centrifuge. Both the supernatant and insoluble fractions were tested for clearing activity.

## Results

*I. Replacement of NaOH by Other Alkalies*

Although it has been well established that addition of sodium hydroxide to pH 10.0 or above will clear suspensions of Gram-negative bacteria treated with lysozyme at pH 3.5 (9, 11, 12), other alkaline compounds had not been tested for their ability to clear suspensions so treated. Therefore, mono- and di-valent cations (KOH, NaOH, $Li_2CO_3$, $NH_4OH$, $Ba(OH)_2$, and $Ca(OH)_2$ were tested to determine if clearing was related more to pH than to the cation of the alkalies.

Results showed that a relationship between pH and clearing existed and that it was logarithmic above pH 7.0. At higher pH values (10.0 and above), the difference between clearing ability of the cations was not as marked as it was at the lower values (pH 7.0). In decreasing order for clearing, the cations at pH 8.0 were: K, Li, Na, $NH_4$, Ba, and Ca.

The difference between maximum (with $K^+$) and minimum clearing (with $Ca^{++}$), however, was seldom more than 10% transmission (T) even at pH 8.0. Therefore, the relationship between clearing and type of cation (although it did exist) was not as marked as might be expected and pH was the more important factor.

*II. Autolytic Enzymes in Lysozyme Bacteriolysis during the Nakamura Treatment*

The possible presence of autolytic enzymes has generally been recognized as helpful in explaining clearing phenomena during lysis of bacterial cells. Warren *et al.* (18) stated "... that an autolytic system influenced the action of lysozyme on lysis of *Ps. aeruginosa*".

Therefore, *Pseudomonas aeruginosa*, *Neisseria catarrhalis*, *Pseudomonas syncyanea*, and *Escherichia coli* were rescreened to determine if an optimal pH for activation of an autolytic system existed. The only conclusion that could be made was that any pH below 5.0 allowed for maximum clearing of all cells in the presence of alkali. Even pH values below 1.5 were found to be satisfactory, providing that lysozyme was added to bacterial suspensions which had been washed free of acid. Apparently, lysozyme is destroyed at pH 2.0 and below, but its essentiality to clearing was obvious.

*Inhibition of Clearing by Heat*

Because no pH optimum for activation of autolytic enzymes could be established, cells were heated to determine if such treatment decreased final clearing. A temperature as low as 75° C. for 1 minute decreased final clearing to alkali with *P. aeruginosa*. Heating also lowered clearing with *P. syncyanea* and *S. cholerasuis*, but *increased* it with *E. coli* and *N. catarrhalis* (Table I). These results were difficult to explain on the basis of autolytic enzyme inactivation unless it was assumed that some of them were thermolabile and others thermostable.

TABLE I

INHIBITION OF LYSOZYME BACTERIOLYSIS BY HEAT

Temperature 100° C.

| Organism | Heating time, min. | % transmission | | |
|---|---|---|---|---|
| | | Zero | 30 min. | After alkali |
| *Pseudomonas aeruginosa* | 0 | 14 | 8 | 91 |
| *Pseudomonas aeruginosa* | 10 | 14 | 8 | 47 |
| *Pseudomonas syncyanea* | 0 | 15 | 12 | 51 |
| *Pseudomonas syncyanea* | 10 | 12 | 8 | 40 |
| *Salmonella cholerasuis* | 0 | 13 | 11 | 46 |
| *Salmonella cholerasuis* | 10 | 16 | 12 | 44 |
| *Escherichia coli* | 0 | 10 | 9 | 20 |
| *Escherichia coli* | 10 | 10 | 7 | 30 |
| *Neisseria catarrhalis* | 0 | 11 | 14 | 53 |
| *Neisseria catarrhalis* | 10 | 10 | 13 | 68 |

Because heating lowered the amount of clearing by alkali with some organisms, it could have been assumed that autolytic enzymes were being inactivated. Such experiments are reported as evidence to support autolytic enzyme action by workers who observe decreased clearing of cellular suspensions after heating (1, 5, 13, 17, 18). Experiments designed to demonstrate

that suspensions of heated cells would clear to a greater extent when they were incubated with homologous live cells (transfer or release of autolytic enzymes?) and lysozyme were unsuccessful.

*Effects of Inhibitors*

Compounds (iodoacetic acid, iodosobenzoic acid, 2,4-dichlorophenoxyacetic acid, potassium oxalate, dicyandiamide, sodium fluoride, sodium phenylphosphate, 2,4-dinitrophenol, and potassium cyanide) which might specifically inhibit autolytic enzymes were also tested with live cells or cells frozen and thawed three times. Potassium cyanide permitted increased clearing instead of inhibition. Investigation showed that a pH of 9.8 existed before addition of alkali. Since KCN is known to undergo partial decomposition, yielding KOH and HCN, the net effect was a Nakamura-type reaction. All other compounds except iodosobenzoic acid showed little or no effect on final clearing in alkali. Iodosobenzoic acid, however, did inhibit final clearing by NaOH.

*Tests of Cell-free Systems*

Further studies were attempted to determine if autolytic enzymes responsible for clearing activity could be demonstrated in cell-free preparations obtained from *P. aeruginosa* or *E. carotovora*. The cell-free preparations were tested using as substrate homologous or heterologous Gram-negative species (*P. aeruginosa*, *E. carotovora*, *E. coli*, *S. cholerasuis*, *N. catarrhalis*) which had been heat-treated at different temperatures for varying lengths of time (5 to 60 minutes at 100° C. or 15 seconds to 15 minutes at 75° C.). None of the different types of cell-free preparations increased clearing with *any* of the organisms tested.

Sequential addition of lysozyme and/or the cell-free preparations to heated cell suspensions also showed no increased clearing. Addition of ferrous iron and cysteine (to protect —SH enzymes) together appeared to aid clearing (e.g., 8% T more clearing with *E. coli* and 5% T more clearing with *P. aeruginosa*), but the results were inconsistent and could not be confirmed when adequate controls were used.

The washed residues left after freeze–thaw extraction were also exposed to lysozyme with or without Nakamura conditions to determine if the "autolytic" enzymes were still present, i.e., not extracted. Without exception, they behaved like live and intact cells. Heating of such preparations prior to lysozyme exposure always resulted in the usual lowered clearing in the presence of alkali.

Precipitation, therefore increased opacity of cell-free preparations at pH 3.5, interfered with light readings, but pre-precipitation of these materials at pH 3.5 also failed to improve subsequent clearing activity. These studies did show that a lowered pH caused coagulation and precipitation of cell substances with a consequent decrease in % T. In that way, they confirmed electron microscopic observations showing increased electron opacity of treated cells (pH 3.5) prior to alkali addition (9).

*Inhibition of Clearing by Metals*

In an attempt to increase activity of the cell-free preparations, salts of metallic ions were screened. The compounds included $ZnCl_2$, $CaCl_2$, $FeSO_4$, $MgSO_4$, and $Al_2(SO_4)_3$ at a concentration of $1.6 \times 10^{-3}$ *M*. They were tested with cells of *E. coli* or *P. aeruginosa* in the presence of a cell-free preparation obtained by freeze–thaw extraction of *P. aeruginosa* with saline. The results obtained when *P. aeruginosa* was used are shown in Table II.

TABLE II

SALTS OF METALLIC IONS AND BACTERIOLYSIS OF *P. aeruginosa*

| Salt | % Transmission | | |
|---|---|---|---|
| | Zero | 1 hr. | After alkali |
| None | 19 | 18 | 61 |
| $ZnCl_2$ | 18 | 18 | 23 |
| $CaCl_2$ | 17 | 18 | 30 |
| $FeSO_4$ | 16 | 17 | 62 |
| $MgSO_4$ | 17 | 17 | 48 |
| $Al_2(SO_4)_3$ | 17 | 18 | 20 |

All cations except ferrous iron decreased rather than increased the amount of clearing obtained. Ranking them according to increasing order of inhibition, the cations were Mg, Ca, Zn, and Al. Decreased clearing of cells in the presence of metal cations has been reported elsewhere (16).

Because metals are often chelated by natural compounds (particularly proteins), it appeared possible that less clearing occurred because of formation of chelate-type compounds having decreased solubility rather than because of inhibition of autolytic enzymes. This was tested by adding the metallic ions to clear but viscous cell-free supernatants obtained by freeze–thaw extraction of *P. aeruginosa*. Lowering the pH (3.5) caused them to precipitate and lose viscosity as expected. Usually, such precipitates were immediately cleared by raising the pH to 10.0. After incubation (45° C. for $\frac{1}{2}$ hour) with the salts of the metallic ions, however, decreased clearing with alkali was again observed except in the tubes containing ferrous iron. Thus we find that in intact cells or in cell-free preparations, the action of the metallic ions was to decrease solubility of cytoplasmic constituents in alkali.

*Inhibition of Clearing by Heat and Iodosobenzoic Acid after Lysozyme Exposure*

The studies with metallic ions prompted further studies regarding the mechanisms responsible for the decreased clearing that occurred with heated cells or cells treated with iodosobenzoic acid. Investigations showed that heating *after* the supposed autolytic reactions had taken place *also* greatly

TABLE III

INFLUENCE OF HEAT ON CLEARING OF *P. aeruginosa* AFTER LYSOZYME TREATMENT

| Tube No. | % transmission | |
|---|---|---|
| | Before alkali | After alkali |
| 1 | 15 | 83 |
| 2 | 15 | 41 |

Tube 1 (control). Reacted with lysozyme at pH 3.5 at 45° C. for 30 min. No heating.
Tube 2. Reacted with lysozyme at pH 3.5 at 45° C. for 30 min., then heated for 2 min. at 100° C. before addition of alkali.

decreased clearing in alkali (Table III). Furthermore, addition of iodosobenzoic acid *after* the supposed autolytic enzymes had acted also decreased clearing (Table IV). Therefore, although either heating, the addition of metal cations, or iodosobenzoic acid reduced alkali clearing, none of the reduced clearing could be attributed to destruction or inactivation of autolytic enzymes.

TABLE IV

INFLUENCE OF IODOSOBENZOIC ACID ON CLEARING OF *P. aeruginosa* AFTER LYSOZYME TREATMENT

| Tube No. | % transmission | | |
|---|---|---|---|
| | Zero | 30 min. | After alkali |
| 1 | 22 | 15 | 85 |
| 2 | 22 | 15 | 72 |
| 3 | 23 | 16 | 68 |

Tube 1. No IBA. Reaction proceeded to normal conclusion.
Tube 2. IBA added at start of experiment.
Tube 3. IBA added at the end of 30 min. and before addition of alkali.

### *III. Replacement of Hydrochloric Acid by Organic Acids*

Since it was not definitely known which was the more important factor, a low pH or the type of acid, different acids were screened at several pH values for their ability to condition cells for good clearing in the presence of alkali. Because inorganic acids are more powerful hydrolyzing agents and since non-specific hydrolysis of cellular constituents might have been partially responsible for the clearing reaction, organic acids (acetic, formic, lactic, and butyric) were tested. Various dilutions were used to obtain different pH values and the final clearing was compared to a control set of figures obtained using hydrochloric acid. Typical results are shown in Table V for cells incubated at or near pH 3.6.

Similar data also obtained with *P. aeruginosa* and *P. syncyanea* were interpreted to mean that good clearing could be obtained with any of the acids tested, provided that the pH of the suspensions was below 5.0.

TABLE V

CLEARING OF CELLS IN THE PRESENCE OF ORGANIC ACIDS

| Organism | Acid | pH | % transmission | |
|---|---|---|---|---|
| | | | Before alkali | After alkali |
| *S. cholerasuis* | HCl | 3.6 | 27 | 92 |
| | Formic | 3.6 | 22 | 94 |
| | Acetic | 3.6 | 36 | 96 |
| | Lactic | 3.5 | 32 | 98 |
| | Butyric | 3.6 | 33 | 95 |
| *N. catarrhalis* | HCl | 3.5 | 26 | 95 |
| | Formic | 3.6 | 29 | 97 |
| | Acetic | 3.6 | 38 | 97 |
| | Lactic | 3.5 | 40 | 99 |
| | Butyric | 3.5 | 34 | 98 |

*Lysis with Neutral or Acid Conditions of pH*

Although a low pH during incubation with lysozyme was an important factor in the clearing reaction, it appeared necessary to see if some of the more sensitive bacteria would undergo good lysis at neutrality without the addition of alkali. Warren *et al.* (18) reported that *P. aeruginosa* was easily lysed by lysozyme at neutral pH, and the suggestion seemed evident that the Nakamura treatment was not needed to demonstrate the presence of the lysozyme substrate in Gram-negative bacteria. Fig. 1 shows that *P. aeruginosa* lysed well at neutral pH. Further, good lysis could be obtained

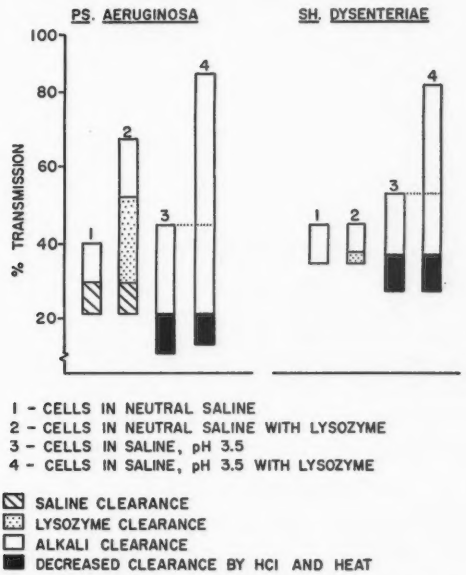


FIG. 1. Neutral vs. acid lysis of certain Gram-negative bacteria.

with *Neisseria perflava* under the same conditions. Other Gram-negative bacteria, however, showed little or no response to lysozyme without the Nakamura treatment. This phenomenon is demonstrated in Fig. 1 with *Shigella dysenteriae.*

Therefore, preliminary incubation at the low pH with lysozyme was not only necessary to obtain *good* clearing, but with *all* organisms tested, a *greater* clearing was recorded when the Nakamura treatment was employed. Therefore, the suggestion of Warren *et al.* (18) cannot be used to generalize with regard to all Gram-negative bacteria.

*Pretreatment of Cells with Acid*

Although acid was necessary for good final clearing by alkali, it was not known whether the acid had to be present *with* lysozyme for maximal action. Therefore, cells were treated with acid (hydrochloric acid at pH 3.5 at 45° C.) for ½ hour after which the acid was removed by several washings in neutralized saline before lysozyme was added in a neutral system. In every case, the suspensions manifested good clearing when alkali was added.

Since lysozyme could act at neutral pH after acid pretreatment it appeared necessary to see if the addition of acid after lysozyme action at neutral pH would also allow good clearing. Typical results are given in Table VI for an experiment performed at 45° C. The same type of response was obtained when cell suspensions were treated at room temperature (25° C.).

TABLE VI

ACID VS. LYSOZYME PRETREATMENT WITH GRAM-NEGATIVE BACTERIA

| Organism | Tube No. | % transmission | | |
|---|---|---|---|---|
| | | Zero | 30 min. | After alkali |
| *E. carotovora* | 1* | 11 | 8 | 23 |
| *E. carotovora* | 2† | 10 | 11 | 66 |
| *S. gallinarum* | 1* | 14 | 12 | 27 |
| *S. gallinarum* | 2† | 12 | 13 | 83 |

*Pretreated with lysozyme in a neutral system before removal of lysozyme and addition of HCl to pH 3.5.

†Pretreated with HCl at pH 3.5 before lysozyme was added to a neutralized system.

NOTE: Final pH in all tubes was 10.0.

Because subsequent addition of acid following lysozyme treatment at neutral pH did not permit good clearing, further studies were performed using three successive exposures as follows (cells always washed in saline and then made to volume between each 15 minute exposure at 45° C.):

*S. gallinarum* (INITIAL % T = 22)

| | | |
|---|---|---|
| First exposure: | Lysozyme at neutral pH | |
| Second exposure: | Hydrochloric acid at pH 3.5 | |
| Third exposures: | Neutral lysozyme (to pH 10.0) | Neutral saline (to pH 10.0) |
| Final % T | 70 % T | 41 % T |

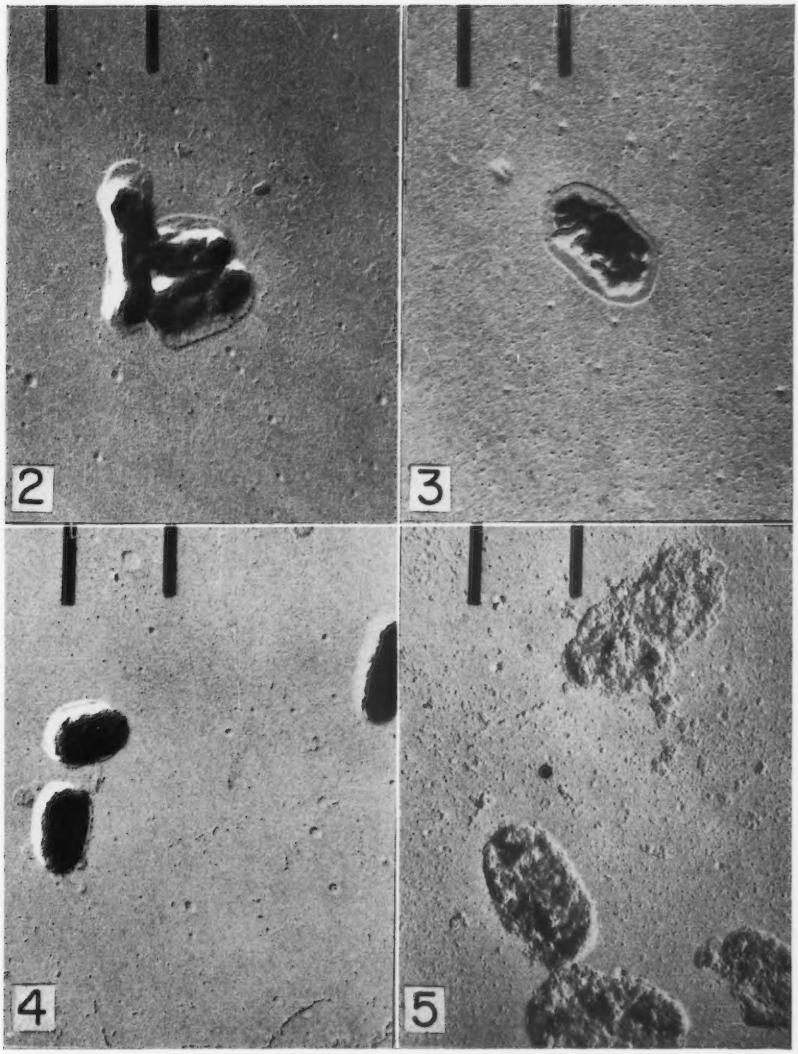


FIG. 2. *E. carotovora.* Treatment: 1 hr. at 45° C. in neutral saline. NaOH added to pH 10.2. % T, 32.
FIG. 3. *E. carotovora.* Treatment: 1 hr. at 45° C. in neutral saline with lysozyme. NaOH added to pH 10.2. % T, 34.
FIG. 4. *E. carotovora.* Treatment: 1 hr. at 45° C. in saline at pH 3.5. NaOH added to pH 10.2. % T, 39.
FIG. 5. *E. carotovora.* Treatment: 1 hr. at 45° C. in saline at pH 3.5 with lysozyme. NaOH added to pH 10.2. % T, 78.
Markers at top of pictures denote magnification; distance between markers is 1 micron.

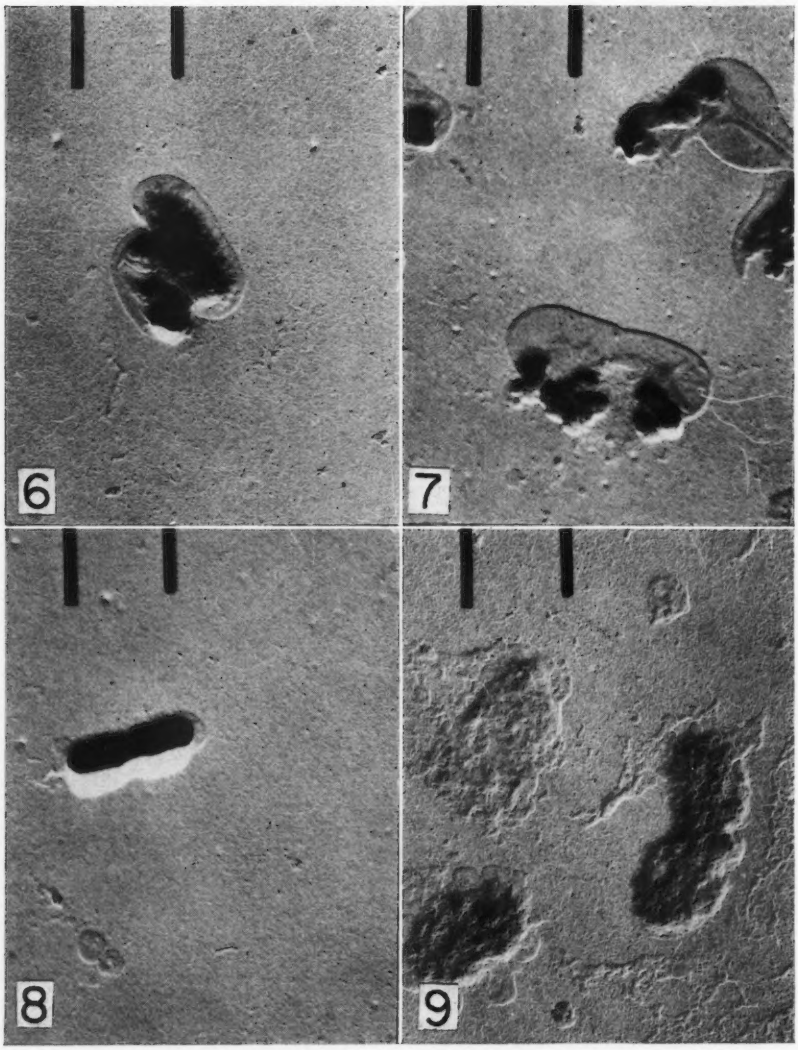


FIG. 6. *S. gallinarum.* Treatment: 1 hr. at 45° C. in neutral saline. NaOH added to pH 10.2. % T, 34.
FIG. 7. *S. gallinarum.* Treatment: 1 hr. at 45° C. in neutral saline with lysozyme. NaOH added to pH 10.2. % T, 36.
FIG. 8. *S. gallinarum.* Treatment: 1 hr. at 45° C. in saline at pH 3.5. NaOH added to pH 10.2. % T, 43.
FIG. 9. *S. gallinarum.* Treatment: 1 hr. at 45° C. in saline at pH 3.5 with lysozyme. NaOH added to pH 10.2. % T, 79.
Markers at top of pictures denote magnification; distance between markers is 1 micron.

These data demonstrated that acid treatment had to precede lysozyme exposure before good clearing could be obtained with alkali. An uncovering or a dissociation of the lysozyme substrate from a "bound" state at the low pH was strongly suggested.

*IV. Further Microscopic Studies of Clearing*

Previous experimentation had shown that addition of alkali to cells treated with lysozyme at a neutral pH caused very little clearing (Fig. 1). However, if the cells were exposed to lysozyme in an acid environment or after acid pretreatment, clearing with alkali was increased. Therefore, it appeared that the lysozyme substrate was either covered by, or complexed with, other substances within the cell wall. Cells were observed with the electron microscope to see if changes consistent with this viewpoint could be demonstrated.

Each set of duplicate bacterial suspensions (*Salmonella gallinarum* and *E. carotovora*) at pH 3.5 or neutral pH was treated with lysozyme in saline for 1 hour at 45° C. Control tubes (no lysozyme), which were tested simultaneously, were also adjusted either to pH 3.5 or neutrality. Sodium hydroxide was added to all tubes to pH 10.2 at the end of 1 hour. The % T of all tubes at zero time was approximately 20. The final % T is recorded with the electron micrographs in Plates I and II to show a comparison between extent of cell damage and spectrophotometric changes.

When incubation was at neutral pH, addition of alkali caused little increase in % T either in the presence or absence of lysozyme (Figs. 2, 3, 6, and 7). Also, cell damage was not extensive except for coagulation and shrinking of the cytoplasm away from the cell wall. When the cells were incubated with lysozyme in the presence of acid, however, addition of alkali caused a sharp rise in % T. Morphologically, the cells appeared less dense and swollen, and several were dispersed (Figs. 5 and 9). Another very obvious difference was the absence of any intact cell walls in the preparations. (Material resembling cell wall debris was evident in some preparations.) These reactions were very striking and the pattern of damage to the cells was very similar for all cells treated with lysozyme at pH 3.5. Very little or no observable damage to the cell wall occurred in the control mixtures when alkali was added (Figs. 4 and 8).

## Discussion

Because clearing of cellular suspensions was the end point observed after the Nakamura treatment, some consideration of factors conducive to clearing is necessary. Generally, two important factors appeared to be operating: (1) some disruption of the intact cells and/or leaching by alkali; (2) hydration of cellular protein. The latter occurred since addition of alkali caused the cells to swell with a concomitant increase in viscosity (6, 9). This is consistent with the views of Brown (4), who indicated that hydration of proteins is dependent on two factors: (1) size of the water shell carried by the cations; (2) charge on the molecules.

The cells appeared more dense and generally smaller after incubation at pH 3.5 for 1 hour at 45° C. with or without lysozyme. Apparently, the acid and this temperature caused some protein denaturation and precipitation. Since proteins usually precipitate at their isoelectric point, it may be assumed that many of the cell proteins were at or near their isoelectric point (10). The cell suspensions reflected the change by a drop in per cent light transmission. Addition of alkali always resulted in some clearing. Apparently, the cell proteins were raised above their isoelectric point, and the charge became predominantly negative. Such a negative charge would permit an excellent situation for association with a positively charged cation such as sodium. If denaturation and/or some hydrolysis also occurred at the acid and alkaline pH ranges, then the situation would be even better suited for association of cations and protein since intramolecular migration by the cations could occur more easily. Once the cation came close to the protein, hydration of the protein would occur because of the water carried by the cation (water shell). The addition of such water would tend to change the index of refraction of the system to that of water, and clearing would result.

Conversely, again lowering the pH (3.5) would establish H-ion pressures strong enough to permit H-ions to displace the sodium ions, for example, bringing about a balancing of the charges on the protein (near or at its isoelectric point). Displacement of sodium and its water shell and balance of charges would again cause the protein to precipitate with a concomitant drop in per cent transmission and in increased cell density.

Such an argument fits a theoretical system wherein acid and alkali can, in turn, be in intimate contact with all molecules in the system. However, these studies, and other work reported by us (8), demonstrated that some cell wall digestion by lysozyme must occur before extensive contact between cytoplasm and alkali is possible. Therefore, the mechanism relating to clearing of bacterial cells by alkali (Gram-positive and Gram-negative) must be expanded to include lysozyme digestion of the cell wall except in atypical organisms such as *Vibrio cholerae* (Chicago).

Another factor must also be considered in most Gram-negative bacteriolytic systems: acid must be present during lysozyme treatment or it must precede lysozyme exposure. Although it was demonstrated that lysozyme could act on Gram-negative bacteria at a neutral pH, it appeared that acid conditions were necessary for either uncovering the lysozyme substrate or for releasing it from a "complexed" state within the cell wall.

The need for exposing the lysozyme substrate in Gram-negative bacteria by pretreatment complicates the lytic picture. Presumably, it is not possible to say that an organism does not contain the lysozyme substrate merely because the cells fail to lyse at a neutral pH. Testing for the substrate should entail proper use of pretreatment techniques before a negative conclusion can be made.

Although no experimental findings are available to explain the nature of the covering or complexing material, the investigations of other workers

also point to the appropriateness of such an interpretation. Becker and Hartsell (2, 3) demonstrated that the combined action of lysozyme and trypsin (synergistic) greatly increased clearing of heat-killed cells. These investigators stated that protein complexing with the lysozyme substrate could occur. It has also been demonstrated (3, 18) that pretreatment with fat solvents such as acetone greatly increases final clearing. Considering these and other reports (14, 15, 19, 20), it is possible that the substrate is covered by or complexed with a lipoprotein.

Therefore, lysis of Gram-positive and Gram-negative bacteria by lysozyme differs because of the relative availability of the lysozyme substrate in the cell wall of most Gram-negative bacteria. Sensitive Gram-positive species (*M. lysodeikticus* and *Bacillus megaterium*) contain considerable amounts of available substrate in their cell walls, therefore extensive digestion of them by lysozyme will occur at a neutral or an acid pH. With most Gram-negative bacteria, however, the situation would appear to be different because: (1) the substrate does not make up the major portion of the cell wall, and (2) the substrate when present is complexed or covered and, therefore, unavailable for lysozyme digestion without some form of pretreatment.

Although it was not possible to demonstrate that autolytic enzymes were involved during the clearing process, a few comments appear pertinent. It is well known that proteins are more difficultly soluble after heating, yet investigators have chosen instead to interpret decreased clearing after heating of bacterial cells to destruction of autolytic enzymes (13, 18). Our studies have shown that heating of cells after the supposed autolytic enzymes had acted still greatly decreased clearing. Further, it was demonstrated that decreased clearing also occurred when some salts of metallic ions or at least one organic inhibitor (iodosobenzoic acid) were added after the supposed autolytic enzymes had acted. Therefore, until agents presumed to destroy or inactivate autolytic enzymes can be shown to exert their influence on cells without manifestation of physical changes in their subsequent solubility, evaluation of evidence for the presence of autolytic enzymes in systems that show clearing should be made with extreme caution.

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## ADDENDUM

After these papers had been accepted for publication, an article appeared by R. Repaske (Biochimica et Biophysica Acta, 22, 189–191 (1956)) relating to the lysis of Gram-negative bacteria by lysozyme and Versene. Repaske states that "The effect of the chelating agent, Versene, in promoting lysis by lysozyme indicates a hitherto unsuspected role of trace metals in the action of lysozyme on bacteria".

Although we had not considered the possibility of trace metal complexing within the cell walls of Gram-negative bacteria, it is pertinent to refer to the following statement which appears in Section 1, page 6 of "The Versenes", Technical Bulletin No. 2 published by The Bersworth Chemical Company, Framingham, Massachusetts: "Greases, fats and oils are easily removed from all surfaces by the use of Versene. Versene is sufficiently alkaline and has sufficient buffer action to saponify fats and oils. Proteins are easily hydrolyzed and dissolved by Versene".

# QUALITATIVE STUDIES OF SOIL MICROORGANISMS

## XIV. SPECIFIC VITAMIN REQUIREMENTS OF THE PREDOMINANT BACTERIAL FLORA[1]

A. G. Lochhead and Margaret O. Burton


## Abstract

A relatively high proportion of the indigenous bacteria of a field soil (27.1%, corresponding to 14.1 millions/g.) required one or more vitamins for growth. The vitamins found to be essential, either alone or with others, were, in order of frequency, thiamine, biotin, vitamin $B_{12}$, pantothenic acid, folic acid, nicotinic acid, and riboflavin. In all, 16 different 'patterns' were noted for vitamin requirements, the number of vitamins needed by individual strains ranging from one to five. The findings point to the soil as an important habitat of vitamin-requiring bacteria, many of which show potentiality as assay organisms. Their occurrence in the numbers found indicates that growth-factors should receive equal emphasis with antibiotics in problems involving the microbial equilibrium in soil and interrelationships between the normal soil microflora, soil-borne disease organisms, and growing plants.


## Introduction

Soil contains microorganisms of the most diversified characteristics, whether judged on the basis of morphology, physiological properties, nutritional characters, or synthetic abilities. Most of the B-vitamins, as well as other microbial growth factors, are known to be present in fertile soil (1, 5, 8, 9, 10). The presence of vitamins is attributable to release from vitamin-containing plant and animal residues, to liberation from the roots of growing plants, and, probably most important, to synthesis by microorganisms. It is therefore not surprising that bacteria are present for which certain vitamins are essential for growth.

It has been previously shown (6, 11) that among the indigenous soil bacteria are forms requiring one or more preformed vitamins. Subsequent reports (3, 4, 7) have shown the need of various types, more fully described, for certain specific factors, namely vitamin $B_{12}$ and the terregens factor (TF). However, apart from a recent report (5) on the incidence of $B_{12}$- and TF- requiring bacteria in soil and in the rhizosphere of certain plants, no information is available concerning the abundance in soil of bacteria requiring specific vitamins for growth.

The purpose of the present study was to examine the specific B-vitamin requirements of the indigenous soil bacteria and to note the abundance of forms requiring the various growth factors.

## Material and Methods

*Source of Cultures*

Five replicate samples of soil were taken from a field supporting a crop of barley, sufficiently distant from the plants to exclude any rhizosphere

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Contribution No. 421 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

effect. Appropriate dilutions were plated on plain soil extract agar without added energy material, chosen as being the least selective medium for the indigenous soil bacteria. It was prepared by autoclaving 1 kg. of field soil with 1 liter of tap water for 20 minutes at 15 lb., filtering, and bringing the volume to 1 liter. To the filtrate were added 0.02% $K_2HPO_4$ and 1.5% agar, with final pH = 6.8. After 14 days' incubation at 26° the plates were counted and from suitable plates or sectors all colonies were picked (approximately 100 for each replicate) and stab inoculations made into soil extract semisolid (soil extract plus 0.02% $K_2HPO_4$, 0.1% yeast extract, 0.3% agar) to serve as stock cultures for further study. In all, 499 cultures were so isolated from the five replicate samples and considered as a group for determination of their vitamin requirements.

*Vitamin Requirements*

The basal medium consisted of inorganic salts, glucose, and vitamin-free 'Casamino acids'. It was prepared by adding to 1 liter of distilled water: $K_2HPO_4$, 1.0 g.; $KNO_3$, 0.5 g.; $MgSO_4.7H_2O$, 0.2 g.; $CaCl_2$, 0.1 g.; NaCl, 0.1 g.; $FeCl_3.6H_2O$, 0.01 g. The solution was adjusted to pH 6.8, heated to boiling, cooled, and filtered; 1.0 g. glucose and 1.0 g. 'Casamino acids' were then added.

Determinations of the vitamin needs of the isolates were carried out in three stages. Each culture was first inoculated into the basal medium and into the basal medium plus all the vitamins. By this means all organisms capable of growing in the former, and thus not requiring vitamins, were eliminated. Those that responded to the complete vitamin mixture were submitted to a further test in which the vitamins were divided into two groups. The growth responses were noted upon the addition of each group, as well as of both groups, to the basal medium. In this way it was possible to eliminate, in many cases, a group of vitamins found to be non-essential. Depending upon the results of the group tests, the detailed requirements of the cultures were determined by preparing a series of appropriate media containing, respectively, the group or groups with essential factors and the same combinations minus each vitamin in turn. Basal medium controls were included in all test series.

The vitamins, with the amounts at which they were used per 100 ml. of medium, were as follows:

*Group A.*—thiamine, 50 μg.; calcium pantothenate, 50 μg.; biotin, 0.1 μg.; vitamin $B_{12}$, 0.2 μg.; folic acid, 10 μg.

*Group B.*—riboflavin, 50 μg.; pyridoxine 50 μg. (with pyridoxal, 50 μg.; and pyridoxamine, 10 μg.); *p*-aminobenzoic acid, 50 μg.; nicotinic acid, 50 μg.; choline, 2 mg.; inositol, 5 mg.

The various liquid media, dispensed in test-tubes, were inoculated by loop transfer (1 mm.) from the semisolid stock cultures. Growth responses were normally read after 5 days' incubation at 26° C. though a small percentage of more slowly growing organisms were kept longer. A vitamin was considered

essential when no growth occurred on its omission from an otherwise adequate medium. In doubtful cases the tests were repeated and, if necessary, additional serial transfers made to eliminate any possible carryover effect.

## Results

The main findings are summarized in Tables I and II. Of a total of 499 isolates, 135 or 27.1% required one or more vitamins for growth. On the basis of an average total bacterial count of 52.4 millions/g. for the five replicate samples examined it was estimated that one or more vitamins were essential for the development of 14.1 millions/g. of soil.

The vitamins found to be needed, either alone or with others, are shown in Table I in order of frequency, together with the estimated numbers of bacteria per gram of soil requiring them. With the majority of the strains (63%), more than one vitamin was required; only in the case of thiamine, biotin, and riboflavin were organisms found that needed but a single vitamin, representing, for the riboflavin group, all that gave response to this factor. Thiamine was the most frequently required vitamin with 19.2% of the total isolates finding it essential; the results thus provide further evidence of the need for this vitamin so frequently encountered. Biotin, as expected, proved to be likewise prominent among the growth factors required. However, of special interest was the finding of $B_{12}$ as the third most commonly required vitamin, the results confirming previous studies (5, 7) that pointed to soil as an important habitat of vitamin $B_{12}$-requiring organisms.

For none of the isolated cultures was pyridoxine, *p*-aminobenzoic acid, choline, or inositol found to be essential though in some cases these factors stimulated growth. It is likely that bacteria requiring one or more of these factors occur in soil; however, the results suggest that their numbers, estimated in the present study at less than 100,000/gm., are relatively small.

TABLE I

VITAMIN REQUIREMENTS AND INCIDENCE OF BACTERIA RESPONDING TO VARIOUS GROWTH FACTORS

| Vitamin required | Alone | With others | Total | % of total isolates | Approximate number/g. soil |
|---|---|---|---|---|---|
| Thiamine | 26 | 71 | 97 | 19.4 | 10,200,000 |
| Biotin | 21 | 61 | 82 | 16.4 | 8,600,000 |
| Vitamin $B_{12}$ | 0 | 36 | 36 | 7.2 | 3,800.000 |
| Pantothenic acid | 0 | 23 | 23 | 4.6 | 2,400,000 |
| Folic acid | 0 | 15 | 15 | 3.0 | 1,600,000 |
| Nicotinic acid | 0 | 10 | 10 | 2.0 | 1,000,000 |
| Riboflavin | 3 | 0 | 3 | 0.6 | 300,000 |
| Pyridoxine | 0 | 0 | 0 | < 0.2 | < 100,000 |
| *p*-Aminobenzoic acid | 0 | 0 | 0 | < 0.2 | < 100,000 |
| Choline | 0 | 0 | 0 | < 0.2 | < 100,000 |
| Inositol | 0 | 0 | 0 | < 0.2 | < 100,000 |

TABLE II

PATTERNS OF VITAMIN REQUIREMENTS

| No. of cultures | Thiamine | Biotin | $B_{12}$ | Pantothenic acid | Folic acid | Nicotinic acid | Riboflavin |
|---|---|---|---|---|---|---|---|
| 26 | + | − | − | − | − | − | − |
| 21 | + | + | − | − | − | − | − |
| 23 | + | − | + | − | − | − | − |
| 2 | + | − | − | + | − | − | − |
| 11 | + | + | + | − | − | − | − |
| 1 | + | + | − | + | − | + | − |
| 5 | + | + | − | − | − | + | − |
| 5 | + | + | − | + | + | − | − |
| 1 | + | + | − | + | − | + | − |
| 2 | + | + | − | + | + | + | − |
| 21 | − | + | − | − | − | − | − |
| 2 | − | + | + | − | − | − | − |
| 2 | − | + | − | + | − | − | − |
| 8 | − | + | − | + | + | − | − |
| 2 | − | + | − | + | − | + | − |
| 3 | − | − | − | − | − | − | + |
| Total 135 | 97 | 82 | 36 | 23 | 15 | 10 | 3 |

| | | |
|---|---|---|
| Requiring 1 vitamin | = | 50 cultures |
| Requiring 2 vitamins | = | 52 cultures |
| Requiring 3 vitamins | = | 25 cultures |
| Requiring 4 vitamins | = | 6 cultures |
| Requiring 5 vitamins | = | 2 cultures |
| Total | | 135 |

Table II shows the various 'patterns' of vitamin requirements found, together with their frequency. In all, 16 'patterns' were recognized. For the great majority of the organisms, one or two or, less commonly, three vitamins were sufficient, though with small numbers four or even five were required for growth.

## Observations on Selected Cultures

A number of cultures were selected for more detailed examination, chosen to include forms illustrative of the needs for the various seven vitamins, the effects of which were studied quantitatively. A basal salts–glucose–casamino acids medium, as described above, was used and vitamin additions made at concentrations previously indicated. The general procedure consisted in preparing duplicate 10-ml. quantities of each test medium in 50 ml. Erlenmeyer flasks which were inoculated with one drop of a washed suspension of the test organism which had been grown in a medium containing the complete vitamin mixture. The flasks were incubated in a reciprocal shaker at 26° C. for 2 days, or in some cases longer, depending on the culture, following which turbidity readings were made on a Klett–Summerson photometer, allowance being made for uninoculated controls. The series of media used were based on the results of the qualitative tests and thus varied with the culture, according to the vitamin or vitamins whose effects were being tested.

TABLE III

SELECTED CULTURES WITH DIFFERENT VITAMIN REQUIREMENTS—GROWTH RESPONSE IN DIFFERENTIAL MEDIA

| Cult. no. | Vitamin(s) required | Average turbidity (Klett–Summerson) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Basal medium (no vit.) | 11 vitamins | 11 vit. minus riboflavin | Riboflavin alone | 11 vit. minus thiamine | Thiamine alone | 11 vit. minus biotin | Biotin alone |
| 785 | Riboflavin | 6 | 317 | 12 | 312 | — | — | — | — |
| 824 | Thiamine | 47 | 206 | — | — | 43 | 233 | | |
| 637 | Biotin | 3 | 262 | — | — | — | — | 6 | 290 |

| | | Basal medium (no. vit.) | 11 vitamins | 11 vit. minus nicotinic | Biotin, nicotinic, thiamine | Biotin, nicotinic, pantothenic |
|---|---|---|---|---|---|---|
| 630 | Biotin, nicotinic acid, thiamine | 17 | 232 | 36 | 220 | 38 |
| 709 | Biotin, nicotinic acid, pantothenic | 11 | 110 | 28 | 14 | 115 |

| | | Basal medium (no. vit.) | Thiamine, biotin, pantothenic, folic, nicotinic | 5 vit. minus thiamine | 5 vit. minus pantothenic | 5 vit. minus folic | 5 vit. minus nicotinic |
|---|---|---|---|---|---|---|---|
| 727 | Thiamine, biotin, pantothenic, folic acid | 2 | 167 | 19 | 1 | 17 | 144 |
| 619 | Thiamine, biotin, pantothenic, folic acid, nicotinic acid | 0 | 170 | 14 | 2 | 2 | 2 |

| | | Basal medium (no. vit.) | 11 vitamins | 11 vit. minus $B_{12}$ | $B_{12}$, thiamine | $B_{12}$, biotin | $B_{12}$, thiamine, biotin | Factor B, thiamine, biotin |
|---|---|---|---|---|---|---|---|---|
| 539 | Vitamin $B_{12}$, thiamine (Factor B pos.) | 16 | 285 | 13 | 289 | 25 | 302 | 284 |
| 541 | Vitamin $B_{12}$, thiamine (Factor B neg.) | 5 | 278 | 27 | 274 | 27 | 267 | 20 |
| 530 | Vitamin $B_{12}$, thiamine biotin (stim.) (Factor B neg.) | 13 | 283 | 19 | 160 | 25 | 272 | 15 |

The results are shown in Table III. The organisms needing but a single vitamin, No. 785, 824, and 637, show clear-cut requirements for riboflavin, thiamine, and biotin respectively. It is noted that vitamins other than the one in question were neither essential nor stimulatory, the single factor providing as good growth as the complete medium. Cultures No. 630 and 709 (which require biotin) are both seen to need nicotinic acid; however, they differ in that No. 630 requires, in addition, thiamine and No. 709 requires pantothenic acid. The table shows that for both No. 727 and 619, thiamine, pantothenic acid, and folic acid are essential (in addition to biotin) while No. 619 requires nicotinic acid as well.

TABLE IV

PLAN OF EXPERIMENTS ON VITAMIN RESPONSE BY SELECTED CULTURES

| Culture No. | Vitamin | Range | Basal medium |
|---|---|---|---|
| 785 | Riboflavin | 0.005–0.1 μg./ml. | Salts, glucose, Casamino acids |
| 824 | Thiamine | 0.1 –5.0 mμg./ml. | Salts, glucose, Casamino acids |
| 630 | Nicotinic acid | 0.005–0.1 μg./ml. | Salts, glucose, Casamino acids, thiamine, biotin |
| 619 | Pantothenic acid | 0.05 –5.0 μg./ml. | Salts, glucose, Casamino acids, thiamine, biotin, folic acid, nicotinic acid |
| 619 | Folic acid | 0.001–0.05 μg./ml. | Salts, glucose, Casamino acids, thiamine, biotin, pantothenic acid, nicotinic acid |
| 541 | Vitamin $B_{12}$ | 0.025–0.5 mμg./ml. | Salts, glucose, yeast extract (Difco) |

Vitamin $B_{12}$, as well as thiamine, is required by cultures No. 539, 541, and 530, the latter being, in addition, stimulated by biotin, though this is not essential to growth. The table also illustrates differences with respect to the ability of Factor B,* the non-nucleotide portion of the molecule of vitamin $B_{12}$, to replace cyanocobalamin in the nutrition of certain strains. Whereas 539 is able to utilize Factor B, cultures No. 541 and 530 are unable to do so.

A number of the same cultures, having requirements for different vitamins, were studied in more detail with the object of noting their responses to varying concentrations of the vitamins in question. The general procedure was similar to that described above. The strains used, as well as the vitamins tested and the basal media employed, are indicated in Table IV. The composition of the media was based on previous findings, to include vitamins required by the organisms other than that being tested. In the case of culture No. 541, requiring $B_{12}$, yeast extract was used.

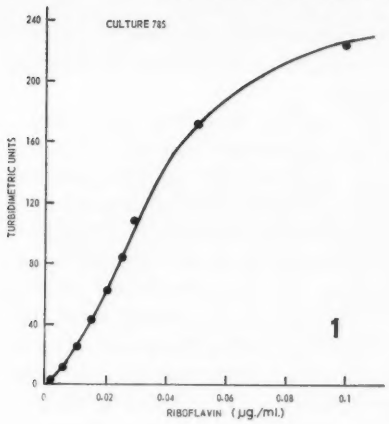
Following preliminary tests to find suitable concentration ranges, growth-response curves in respect to the six vitamins were established as shown in Figs. 1 to 6. Under the experimental conditions the essentiality of the vitamins for growth of the organisms in question was confirmed. The level at which response was evident varied with the factor; however, the effective concentration of a given vitamin might be expected to vary, depending upon the test organism selected.

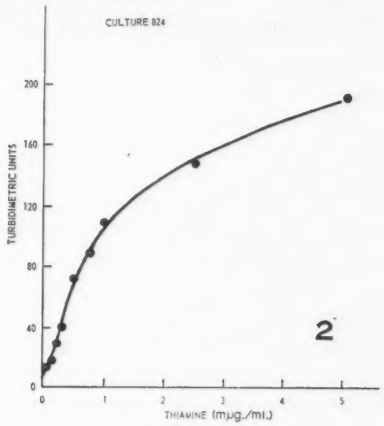
## Discussion

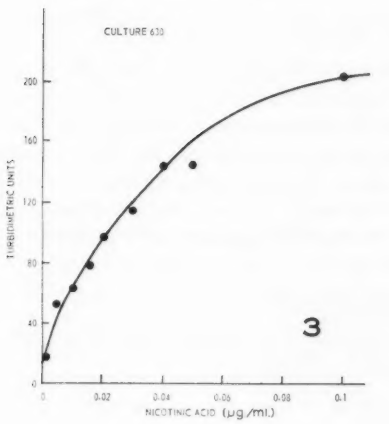
Microbial growth-promoting substances in soil and organisms requiring or synthesizing them have received less attention than the subject of antagonisms. Growth factors may be considered in the same category with antibiotics in so far as they affect the growth of many microorganisms at

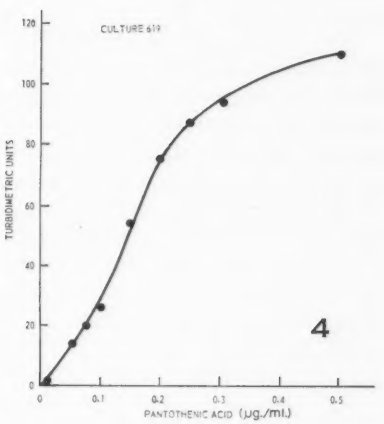
*Kindly supplied by Dr. S. H. Hutner from a sample obtained from Merck and Co., Inc., through courtesy of Dr. David Hendlin.

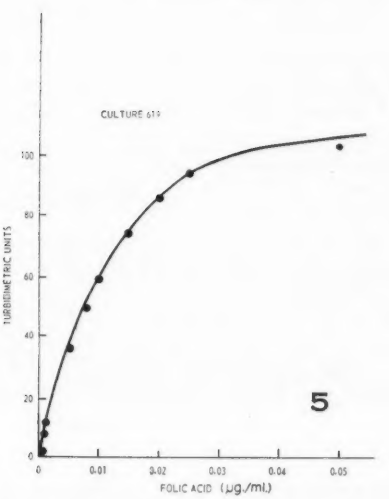
FIGS. 1–6. Response of cultures to six vitamins: Culture No. 785 to riboflavin; No. 824 to thiamine; No. 630 to nicotinic acid; No. 619 to pantothenic acid; No. 619 to folic acid; No. 541 to vitamin $B_{12}$.

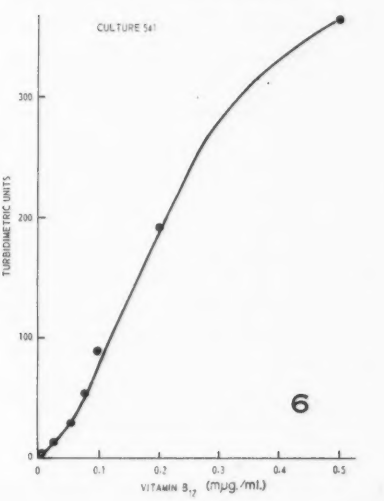

CULTURE 785
TURBIDIMETRIC UNITS
RIBOFLAVIN (µg./ml.)
1


CULTURE 824
TURBIDIMETRIC UNITS
THIAMINE (mµg./ml.)
2


CULTURE 630
TURBIDIMETRIC UNITS
NICOTINIC ACID (µg./ml.)
3


CULTURE 619
TURBIDIMETRIC UNITS
PANTOTHENIC ACID (µg./ml.)
4


CULTURE 619
TURBIDIMETRIC UNITS
FOLIC ACID (µg./ml.)
5


CULTURE 541
TURBIDIMETRIC UNITS
VITAMIN $B_{12}$ (mµg./ml.)
6

very low concentrations and may be produced by microbial synthesis. Consequently growth-factor effects could well be as important as antibiotic activity in affecting the microbial equilibrium.

If the proportion of vitamin-requiring bacteria had been insignificant, their presence might be regarded as of minor importance; however, in view of their occurrence in the numbers found it is apparent that growth-promoting factors should be taken into account in any consideration of the microbial status or potentiality of soil. This appears to be particularly relevant to problems involving the complex interrelationships between the normal soil microflora, soil-borne plant disease organisms, and the plant itself. In such relationships, growth factors, which have been relatively ignored, could have a bearing as significant as that of antibiotics. Indeed it is possible that the lack of progress in applying antibiotic-producing organisms to the control of disease caused by soil-borne pathogens, as recently stressed by Garrett (2), may be attributed in some measure to neglect of the role of factors which promote, rather than suppress, the growth of many soil microorganisms.

The results also emphasize the wide diversity of the soil's indigenous microflora in its nutritional aspects and provide a further example of the interdependence of many of the soil organisms; thus those that require certain specific substances depend upon the synthetic activities of other forms that are less demanding in their nutritional needs. The findings are of some ecological significance in pointing to the soil as an important habitat of vitamin-requiring bacteria, many of them being, as far as known, characteristic of soil and representing hitherto undescribed species with potentiality as vitamin assay organisms.

## Acknowledgment

The authors are indebted to Dr. J. W. Rouatt for making cultures available and to Miss Agnes MacIntosh for technical assistance.

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# THE FEULGEN REACTION IN THE UNSECTIONED MACRO YEAST CELL[1]

G. FRED TOWNSEND


## Abstract

The Feulgen stain was applied to the macro yeast cells, derived from *Saccharomyces cerevisiae*, and Feulgen-positive areas were readily located. Staining could not be observed within the central vacuole and could be found at only one point outside of the vacuole. This point was considered as being the location of the yeast cell nucleus.


## Introduction

The macro yeast cell described by Townsend (4) has been studied with the Feulgen stain. This large cell (derived from *Saccharomyces cerevisiae*), when not sectioned, allows the Feulgen positive areas to be located with considerable accuracy.

## Special Techniques

The dye used in this study was basic fuchsin (Coleman & Bell) of 97% purity, and other chemicals were C. P. grade. The method of DeTomasi (1) was employed when making the Feulgen reagent. The final preparation was filtered through activated charcoal until clear and completely colorless. It was stored and handled in such a manner as to receive a minimum of exposure to air and light. The stock solution, freshly prepared every few days, was kept in a glass stoppered bottle and stored in a refrigerator. Portions of the solution removed for staining were discarded after use.

When working with large cells which are to be mounted whole, special care is required in order to avoid distortion and displacement of internal structures. In consideration of this point it was found that test-tube staining offered the best means of handling the cells. Also desirable was the moderate form of hydrolysis adopted, this step being carried out at a temperature of approximately 25° C. instead of 60° C. Harsh killing and fixing agents were of course avoided. It is important too, that the alcohol, xylol, or balsam additions be made with care, and that ample time be allowed for solution exchange through the cell membranes. Accordingly, after centrifugation and removal of each supernate, the first ½ to 1 ml. of the solution following was introduced slowly from a small-bore glass tube. Single drops were allowed to run down the side of the test tube and gradually mix with the cells at the bottom.

## Methods

A small portion of the liquid yeast culture was placed in a 15 ml. test tube and mixed with an equal quantity of distilled water. After centrifugation the supernate was drawn off with a pipette. The cells were resuspended in

[1]Manuscript received October 10, 1956.
Contribution from the Wahl–Henius Institute, Chicago 14, Ill.

10 ml. of a distilled water rinse and centrifuged. Again the supernate was removed. Then 10 ml. of 40% (vol.) ethanol was added and the cells maintained in suspension for 1 minute. Centrifugation and supernate removal were followed by two more rinses with distilled water.

Hydrolysis was performed at room temperature, which was near 25° C. For this step the cells were held in 10 ml. of *N* HCl for 24 hours. To ensure uniform hydrolysis of the sample, the tube was shaken occasionally. It could be mentioned here that comparable results may be obtained within 6 hours if 10% perchloric acid is substituted for the normal hydrochloric acid.

After removal of the hydrolyzing solution the cells were rinsed twice with distilled water. Ten milliliters of the Feulgen stain was then added and the cells resuspended frequently during a 4 hour period. The temperature was maintained at near 25° C. At the end of this time the stain was centrifuged off and the cells again rinsed twice with distilled water.

Five milliliters of 95% ethanol was added with care and removed after centrifugation. Then followed two successive 5 ml. treatments with 100% ethanol. Five milliliters of xylol was added slowly, centrifuged off, and the step repeated. One-half milliliter of thinned balsam was carefully added to the cells in the tube and the resulting mixture allowed to evaporate to the proper consistency for mounting.

The slides were observed and photographed at 2000×, using a fluorite objective and compensating oculars. The only filter used was a "dalite" filter placed in the substage holder of the microscope.

In this study the counter stain was purposely omitted. It was felt that its non-specific nature might favor uncertainties and cytological misinterpretations. Colored filters were not used because the proper identification of Feulgen-positiveness involves the identification of proper color.

## Results

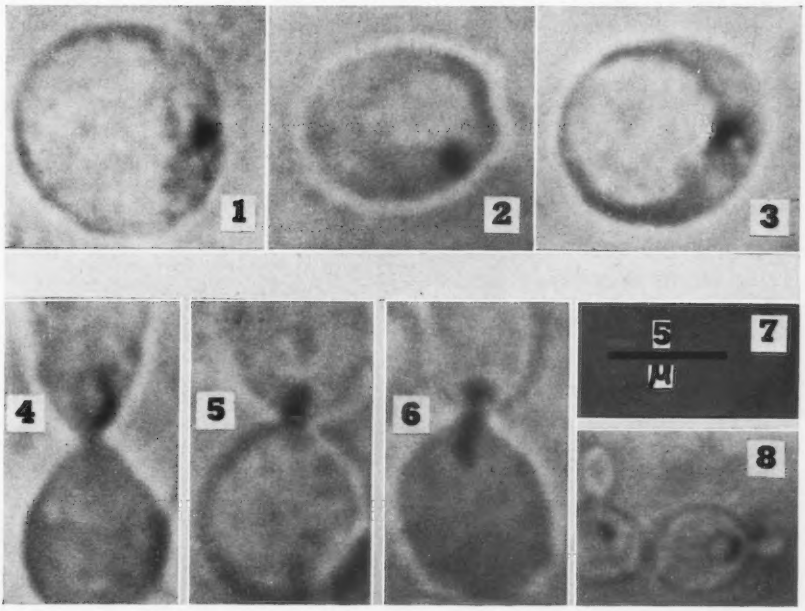
Staining of the yeast cell could be observed only in the area of the large cytoplasmic body. Careful searching of the entire cell revealed no suggestion of staining in any other portion. This finding should be significant for two reasons. First, the appearance of the Feulgen color was not altered by counterstaining or by illumination with colored light. Secondly, it is probable that stained threads as small as $\frac{1}{4}$ micron wide could have been seen, if present, even in cells as wide as 10 microns.

Figs. 1, 2, and 3 are photographs of the macro yeast cell showing the single Feulgen positive area outside the vacuole. Fig. 8, included for comparative purposes, shows normal cells similarly stained. Figs. 4, 5, and 6 demonstrate positions assumed by the Feulgen positive areas during cell division. Fig. 7 is the micron scale for the photomicrographs.

## Conclusions

The results of this study, supported by evidence previously obtained with the Newcomer orcein–carmine stain (3), suggests that the present writer's

PLATE I




Unsectioned yeast cells stained with the Feulgen stain. These cells not counter-stained.

FIGS. 1, 2, and 3. Macro yeast cells with the vacuolar outline and the Feulgen positive area both in the same focal plane, showing the Feulgen positive area outside of the central vacuole.

FIGS. 4, 5, and 6. Macro yeast cells dividing and showing the Feulgen positive areas in different positions.

FIG. 7. Micron scale for the entire plate.

FIG. 8. Normal yeast cells from an actively growing culture, with the same distribution of Feulgen positive material as that in the macro cells.

earlier interpretations of the yeast nucleus should be reconsidered. Since all of the Feulgen positive material found has been located at one point outside of the central vacuole, this location would seem to be the position of the nucleus, an opinion held by most observers. For a recent survey of the ample literature and an interesting discussion of different viewpoints, the reader is referred to Mundkur (2). Also, there is general agreement that the Feulgen positive area, here identified with the nucleus, is quite intimately associated with the chromosomes of the yeast cell. However, cytological details lying beyond the optical limits of the microscope are rather speculative, and their consideration is reserved for future investigations.

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# STAPHYLOCOCCAL TOXINS

## I. FACTORS AFFECTING THE HEMOLYTIC ACTIVITY OF ALPHA TOXIN[1]

A. W. JACKSON AND R. M. LITTLE


### Abstract

The hemolytic activity of α-toxin of *Micrococcus pyogenes* var. *aureus* has been shown to be maximal at pH 6.8–7.0 in phosphate-buffered saline. Within the limits of 25 to 75% hemolysis there is a linear relationship between lysin concentration and extent of hemolysis after incubation for 15 minutes. Citrate and glucose have been shown to inhibit hemolysis by α-toxin. The manner in which the factors studied affect determination of α-toxin activity by a direct hemolysis procedure is discussed.


### Introduction

Although at least three serologically distinct lysins are known to be produced by *Micrococcus pyogenes* var. *aureus*, methods are not available for their separation and little work has been done on their biochemical characterization. For such studies a simple, direct method of estimating the potency of different fractions, obtained under various conditions, is required. Development of a method of this type requires the usual data necessary in establishing enzyme assays, although as indicated by Bernheimer (2), this is not to suggest that the action of the lysin is necessarily enzymatic. Rud (8) has recently reported on the effect of pH and temperature on lysis by the crude toxin produced by a strongly toxigenic strain of *M. pyogenes* var. *aureus* but the identity of the hemolysin was not established. With the exception of the study reported in the above paper no previous investigations of the kinetics of hemolysis by the lysins of this species have been made. Consequently the present experiments were conducted to determine the effects of toxin, red-cell and hydrogen-ion concentration on lysis by α-toxin of *M. pyogenes* var. *aureus*. The kinetics of hemolysis by lysins of a number of other species of bacteria has been studied, however, in an attempt to determine the type of reaction responsible for hemolysis (2, 4) and to develop an accurate method of estimating the potency of the hemolytic toxin (1). Bernheimer (2) divided bacterial toxins into two main groups on the basis of the relationship of rate of hemolysis to toxin concentration. A linear relationship exists between extent of hemolysis and toxin concentration for the theta toxin of *Clostridium welchii*, pneumolysin, tetanolysin, and streptolysin S. These observations are of significance to the present investigation as they indicate the possibility of utilizing degree of hemolysis obtained in a controlled system to determine the potency of hemolysins. There is the further possibility that studies of this kind may assist in characterizing hemolysins of bacterial origin.

[1]Manuscript received July 26, 1956.
Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

## Materials and Methods

*Culture.*—A strain[2] selected in the laboratory for high $\alpha$-toxin production.

*Medium.*—A modification of the medium of Bramann and Norlin (3) was employed for production of toxin. It consisted of 1.5% vitamin-free casein hydrolyzate, 0.5% yeast extract (Difco), and 0.03 *M* dipotassium phosphate, adjusted to pH 7.0. The medium was added in 100-ml. quantities to 500-ml. Erlenmeyer flasks which were then fitted with rubber stoppers through which two glass tubes were inserted, one of which reached to within 1 inch of the surface of the medium, the other providing an outlet for gas. Both tubes were plugged with cotton to provide a seal. These flasks were then sterilized by autoclaving for 20 minutes at 15 lb. pressure.

*Production of toxin.*—Sterile medium prepared as described above was seeded with 1 ml. of a 16 hour culture of the selected strain grown in test tube quantities of the same medium. The flasks were "gassed" for 2 minutes with a mixture of 20% carbon dioxide in oxygen, filtered through a plug of sterile cotton, and incubated at 37° C. for 48 hours with gassing as above morning and evening of each day of incubation. After removal of the cells by centrifuging, the toxin was precipitated and concentrated, by following the first step of the procedure of Whittler and Pillemer (11) and dissolving the precipitate in 10 ml. saline phosphate pH 6.8 (1) per 100 ml. of cell-free medium employed. This preparation is quite stable for 2–3 months at 4° C.

*Purity of $\alpha$-toxin.*—Hemolysis of rabbit erythrocytes by the preparation is completely inhibited by $\alpha$-antitoxin and, in addition, human erythrocytes which are susceptible to hemolysis by $\beta$- and $\delta$- lysin, but not by $\alpha$-toxin are unaffected. These tests indicate that the $\alpha$-toxin employed is free of detectable quantities of other known staphylococcal lysins.

*Erythrocyte suspensions.*—Freshly drawn rabbit blood was immediately mixed with an equal volume of Alsever's[3] solution and stored at 4° C. Aliquots were taken as required, washed three times with physiological saline, and resuspended at the concentration indicated, in either physiological saline or buffered saline. Cells were packed by centrifuging for 10 minutes at 2000 r.p.m. (No. 240 head, International Centrifuge) and resuspended in the required volume of fluid. A 1% cell suspension prepared in this manner contains on the average $1.38–1.4 \times 10^8$ cells/ml.

*Determination of hemolysis.*—Extent of hemolysis was determined by measuring spectrophotometrically the density of hemoglobin in the supernatants after removal of unhemolyzed cells by centrifuging. Since a lapse of time between removal of aliquots for centrifuging and complete sedimentation of the cells cannot be avoided, the procedure was standardized by placing the samples in centrifuge tubes 15–30 seconds before the end of the required period of lysis in order that centrifuging could be begun exactly at the time indicated. Density measurements were made on diluted super-

[2]Selected and kindly provided by A. E. Larner of this Laboratory.

[3]This is a sterile solution containing 2.05 g. dextrose, 0.8 g. sodium citrate, and 0.42 g. sodium chloride per 100 ml. Adjusted to pH 6.1. Obtained from Baxter Laboratories of Canada Limited, Acton, Ontario.

natants (1 ml. in 6 ml., 1% sodium carbonate solution) at 541 mμ (7). Values for total hemolysis were obtained by substituting distilled water for buffer and lysin and proceeding as described above.

*Buffers.*—Isoionic phosphate buffered saline solutions of the desired pH were prepared by combining solutions of monopotassium and disodium phosphate at 0.08 (0.028 *M* $Na_2HPO_4$ and 0.084 *M* $KH_2PO_4$) ionic strength and mixing these buffers with equal volumes of 0.286 *N* sodium chloride (total ionic strength 0.183).

## Results

*Effect of pH on Hemolysis of Red Cells by α-Toxin*

One milliliter aliquots of a 6.5% suspension of rabbit erythroctyes in saline were added to 5 ml. quantities of isoionic buffers at the pH levels indicated in Fig. 1 and allowed to equilibrate for 15 minutes at 37° C. A 0.5 ml. sample of suitably diluted lysin was added to each suspension, thoroughly mixed with the cells, and incubated for 15 minutes at 37° C. Extent of hemolysis was determined as described under "Methods". The results obtained are shown in Fig. 1, B. It will be observed that hemolysis by this toxin is most rapid at pH 6.8–7.0 and decreases quite markedly at pH values above and below the optimum level.

The effect of pH on red-cell volume may influence interpretation of these data, and consequently relative cell volumes were determined in each of the above buffers. One milliliter aliquots of washed rabbit erythrocytes suspended in neutralized saline were added to 5-ml. quantities of the buffers and equilibrated for 15 minutes at 37°. The suspensions were then transferred to Hopkins' vaccine tubes and centrifuged at one time to eliminate variation, and the packed cell volume was recorded. Fig. 1, A. The observed increase

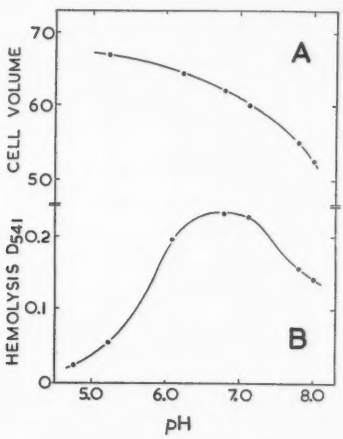


FIG. 1. A. Relative erythrocyte volume at various pH levels.
B. Effect of pH on hemolysis by α-toxin.

in cell volume as pH is lowered is quite marked and is in agreement with the findings of Warburg (10) and of Hampson and Maizels (5). The reduced hemolytic activity of toxin at pH values alkaline to neutral may be partly the consequence therefore of reduced red-cell volume, i.e., lowered turgidity, at these pH levels. Hence the true optimum pH may be somewhat higher than indicated by the curve (Fig. 1, B); nevertheless for practical purposes, employing the buffers as described, maximum hemolytic activity of this lysin will be obtained at pH 6.8–7.0.

*Time Course of Hemolysis at Various Lysin Concentrations*

Various quantities of $\alpha$-toxin suitably diluted in saline, plus saline where necessary to make up to 1 ml., were added to 5-ml. aliquots of a 1.2% suspension of washed rabbit erythrocytes in phosphate buffered saline, pH 6.8, at 37° C. Aliquots were removed at intervals from 5 to 45 minutes and the extent of hemolysis determined as described above. The results of a typical experiment are shown in Fig. 2 where liberated hemoglobin is plotted against time. The shape of these curves indicates that $\alpha$-toxin of *M. pyogenes* var. *aureus* lyses red cells in a manner similar to that of other bacterial toxins (1, 2, 3) and certain other lysing agents of an organic nature (2). When similar data are plotted as shown in Fig. 3, it will be seen that the relationship between extent of hemolysis and toxin concentration is linear over a relatively narrow range of dilutions of the lysin. It may be concluded from an examination of the curves shown in Fig. 3 that a determination of the quantity of hemoglobin released in samples taken after the reaction has proceeded for 15 minutes can be used to measure the activity of the toxin employed provided the extent of hemolysis is greater than 25 and less than 75% of the total.

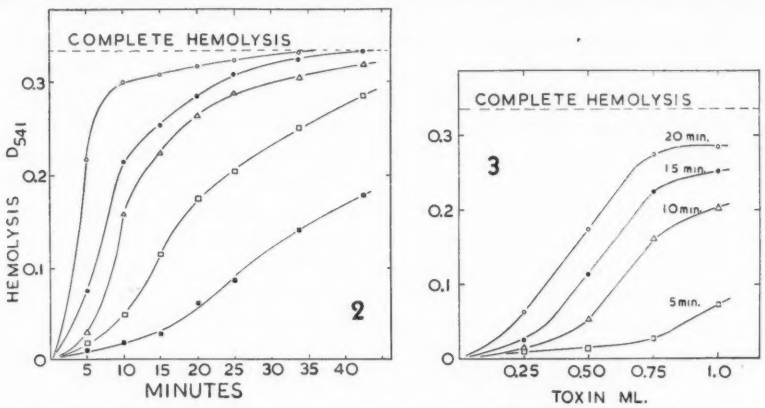


FIG. 2. Time course of hemolysis by $\alpha$-toxin at different concentrations of toxin. Volume of toxin employed beginning with upper curve, 1 ml., double strength, and 1.0, 0.75, 0.5, 0.25 ml. respectively, single strength.

FIG. 3. Extent of hemolysis by $\alpha$-toxin at concentrations shown and after periods of time indicated.

TABLE I

INHIBITION BY CITRATE AND GLUCOSE OF HEMOLYSIS BY α-TOXIN

| Suspending medium | Hemolysis, $D_{541}$ | % inhibition (averages) | Cell volume |
|---|---|---|---|
| Control (phosphate-saline) | 220–210 | 0 | 41–41 |
| Alsever's solution | 57– 54 | 73.5 | 41–41 |
| Citrate* + phosphate saline | 66– 65 | 69.3 | 41–41 |
| Glucose* + phosphate saline | 98– 98 | 54.5 | 41–41 |

*Final concentration: citrate, 6.4 mg./ml.; glucose, 14 mg./ml.

*Effect of Glucose and Citrate on Hemolytic Activity of α-Toxin*

During the course of experiments to determine the effect of pH on the hemolytic activity of α-toxin it was observed that a reference suspension of red cells buffered with Alsevers' solution was much less hemolyzed by the toxin than suspensions at the same pH buffered with phosphate plus saline. In order to determine the effect on lysis of those components of Alsevers' solution not present in the buffers previously used in this study, saline phosphate solutions were prepared to which glucose and citrate were added separately and made up to the same tonicity as the control saline phosphate buffer. The cell volumes and extent of hemolysis by α-toxin when red cells were suspended in these media are shown in Table I. It will be seen that Alsevers' solution, glucose, and citrate all markedly inhibit hemolysis compared with the controls with saline phosphate buffer as suspending medium. Both glucose and citrate are therefore inhibitory to hemolysis by this toxin and should be avoided in suspending fluids used for the determination of α-toxin activity by hemolysis of red cells.

*Effect of Erythrocyte Concentration and Source on Hemolysis by α-Toxin*

The quantities of 6.5% red-cell suspension shown in Table II were added to test tubes and sufficient phosphate-buffered saline (pH 6.8) added to bring the volume to 5 ml. After equilibration at 37°, 0.5 ml. of a suitably diluted toxin was added to each tube and the mixture was incubated for

TABLE II

EFFECT OF ERYTHROCYTE CONCENTRATION ON HEMOLYSIS

| Cells, ml. (6.5% susp.) | Hb total* | Hb released* | % hemolysis |
|---|---|---|---|
| 1.0 | 450 | 410 | 91 |
| 0.9 | 400 | 375 | 94 |
| 0.7 | 330 | 300 | 90 |
| 0.5 | 240 | 220 | 92 |
| 0.3 | 160 | 147 | 92 |

* $D_{541}$, diluted as described under "Methods".

10 minutes. Extent of hemolysis was determined as usual. It will be seen from Table II that over a quite wide range of erythrocyte concentration, $\alpha$-toxin at a uniform level causes hemolysis of a relatively constant proportion of the cells. This indicates that, in determining hemolytic activity of $\alpha$-toxin, red-cell concentration need not be critically exact provided a total hemolysis control is included from which percentage hemolysis may be calculated.

TABLE III

RED-CELL SUSCEPTIBILITY TO HEMOLYSIS

| Rabbit No. | Hb released by toxin | $D_{541}$ total | % hemolysis |
|---|---|---|---|
| 1 | 256 | 308 | 82.5 |
| 2 | 250 | 308 | 81.5 |
| 3 | 256 | 304 | 84.0 |
| 4 | 248 | 308 | 81.0 |
| 5 | 208 | 279 | 74.5 |
| 6 | 166 | 275 | 60.5 |

It was also observed that there is considerable variation in susceptibility to hemolysis between the red cells from different individual animals of a single species (Table III). Thus, comparison of results obtained with erythrocytes from different animals is subject to error. For most studies, adequate cells for a large number of determinations may be obtained from one animal, and thus for practical purposes, the above variability is not a serious hindrance to use of the method.

## Discussion

In order to determine whether a simple method for the determination of $\alpha$-toxin could be devised, based on the recognized hemolytic activity of this toxin, it was necessary to obtain data upon such variables as pH, toxin concentration, cell concentration, time, and suspending fluid, which may affect the rate of hemolysis. Although it is generally recognized that the rate of biological reactions is affected by changes in the pH at which the system operates there is no record of the influence of this variable upon hemolysis by $\alpha$-toxin of *Micrococcus pyogenes* var. *aureus*. In the present study it has been found that a moderately sharp optimum pH about neutrality exists for this reaction and it is apparent that tests conducted at levels above or below pH 6.8–7.0 will give readings which are lower than the maximum obtainable.

With a crude preparation designated "Staphylolysin" Rud (8) obtained quite different curves showing minimum activity at pH 7.2–7.5. The nature of the lysins in this preparation was not determined however, and consequently the conclusion must be drawn that the lysins other than $\alpha$-toxin were present. As indicated above, the preparation employed in the present

study was shown to be free of the other known lysins produced by *M. pyogenes* var. *aureus* and was completely inhibited by $\alpha$-antitoxin.

The demonstration of a linear relationship between hemolysin concentration and extent of hemolysis over the range of 25 to 70% lysis indicates that relative $\alpha$-toxin concentration of different preparation may be obtained by reference to a standard curve provided the percentage of lysis obtained with the unknown falls between the above limits. Burrows (4) obtained similar results with the $\alpha$-toxin of *Clostridium welchii* and concluded that the potency of the toxin could be obtained by measurement of hemolysis under controlled conditions.

The observation that glucose and citrate either singly or in combination inhibit hemolysis by $\alpha$-toxin should be emphasized since the presence of these compounds in the suspending medium would lead to serious errors in analysis by a direct hemolysis method. According to Bernheimer (2), the action of some hemolytic agents is known to be inhibited by sugars, and lysis by *Clostridium septicum* hemolysin was found by the above author (2) to be inhibited by sucrose. It was suggested (2) that the sugar acts by preventing swelling, which, in a number of instances, has been shown to precede liberation of hemoglobin from the erythrocyte attacked by a lytic agent. No explanation can be offered regarding the manner in which citrate inhibits hemolysis.

It is apparent that a standardized suspension of erythrocytes, with regard to numbers, susceptibility to hemolysis, and hemoglobin content would be required in order to relate activity of a hemolysin to absolute amounts of hemoglobin released. Such preparations would be difficult if not impossible to obtain from day to day. Our results indicate that cells taken from different individual animals of one species show quite marked variation in susceptibility to lysis by $\alpha$-toxin. Schwab (9) noted a similar variability in studies with an intracellular hemolysin of streptococci. Furthermore, Albertsen (1) found that cells taken from one horse at intervals varied in susceptibility to lysis by *Corynebacterium pyogenes* hemolysin. Consequently it seems logical to attempt to relate lysin activity to the proportion of cells hemolyzed. It was necessary therefore to determine whether changes in cell concentration markedly affect the proportion of cells hemolyzed by a constant quantity of toxin. The results obtained indicate that rather marked variation in cell concentration is not associated with a significant change in the proportion of cells hemolyzed. This is in agreement with the observation of Marks and Vaughan (6).

In conclusion, it should be emphasized that the data presented shows the extent to which certain variables influence rate of hemolysis, and consequently the valid limits within which extent of hemolysis may be related to concentration of lysin employed. Taking 50% hemolysis under the above conditions as representing an arbitrary unit we are able to determine relative activities of different preparations with sufficient accuracy for present purposes. It will be noted that for most of the studies reported above, presentation of

relative densities of hemoglobin released is adequate. Thus for research purposes, it is possible to determine the relative hemolytic activity of preparations without use of antitoxins, the best of which introduces variables that, in our present state of knowledge, cannot be controlled.

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# IN VITRO PRODUCTION OF ERGOT ALKALOIDS BY CULTURES OF CLAVICEPS PURPUREA (FR.) TUL.[1]

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## Abstract

Stationary cultures of *Claviceps purpurea* (Fr.) Tul. grown 50 days on a liquid medium containing mannitol, ammonium succinate, and salts produced small amounts of ergot alkaloids.

Ergometrine, ergometrinine, ergotamine, ergotaminine, ergocornine, ergocorninine, ergocryptine, agroclavine, and penniclavine were identified by paper chromatography of culture extracts. The selection of strains, the composition of the medium, and the length of the incubation period appeared to be important factors influencing the production of alkaloids.

## Introduction

Since 1930 the production of ergot alkaloids by pure cultures of *Claviceps purpurea* has frequently been reported (8, 9, 10, 13, 14, 15, 18, 19, 22) but in most cases the methods employed for the identification of the alkaloids were not exacting and depended solely upon characteristic biological properties and color reactions. Production, if actually achieved, was at a very low level. Many recent workers (11, 12, 17, 20, 24) have failed to reproduce these results and, in fact, the majority of reports since 1950 have indicated that alkaloids are not produced by the fungus *in vitro*. Abe and his co-workers (1 to 8), and subsequently Stoll and his group (22) using Abe's methods, have, however, demonstrated convincingly that alkaloid production is possible. Where high yields were obtained, the products were found to be not the substituted amides of lysergic acid hitherto isolated from rye sclerotia but new derivatives of the ergoline ring system. Thus Abe's group have found that certain cultures isolated from *Agropyron semicostatum* Nees produced good yields of agroclavine (1), while cultures from *Elymus mollis* Trin. produced substantial quantities of elymoclavine (7). A number of other alkaloids of novel constitution have also been isolated in minor amounts. Stoll *et al.* (22) described the isolation of penniclavine, elymoclavine, and agroclavine from a strain of *Claviceps* found on *Pennisetum typhoideum* Rich. Recently, the Japanese workers (8) have reported paper chromatographic evidence that small amounts of ergometrine, ergotamine, ergosine, ergocristine, ergokryptine, and ergocornine and of alkaloid X, agroclavine, and elymoclavine are produced *in vitro* by a Spanish strain of *Claviceps purpurea*. Strains isolated from 10 different grasses were examined, and all cultures were found to produce agroclavine, elymoclavine, alkaloid X, ergokryptine, and ergosine; two produced ergometrine; three, ergotamine; three, ergocristine; and one, ergocornine.

In view of the importance of ergot alkaloids and the obvious advantages of commercial production in artificial media, an investigation into the possibili-

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ties of *in vitro* production was undertaken. This phase of the study was designed to resolve the conflict between recent reports in the literature concerning the formation or nonformation, in artificial media, of ergot alkaloids of the type derived from lysergic acid.

## Materials and Methods

*Growth Conditions*

The history, nutrition, and method of preparing the inocula of the three strains used in these studies have been described in a previous publication (23). The influence of media composition on production was examined by the use of five different media (Table I).

These media were dispensed either in 40 ml. aliquots in 125 ml. Erlenmeyer flasks, or 50 ml. in 250 ml. Erlenmeyer flasks. Shaken cultures were grown as already described (23); stationary cultures were incubated at 27° C. either in the dark or diffuse natural light.

*Estimation of Yield of Alkaloid*

Cultures were selected at intervals, separately homogenized in a Waring blendor, and freeze-dried. They were then shaken for four hours with peroxide-free ether (50 ml.) containing concentrated ammonium hydroxide (0.3 ml.), filtered, washed with fresh ether (20 ml.), and the filtrate concen-

TABLE I

COMPOSITION OF MEDIA

| | Concentration per liter | | | | |
|---|---|---|---|---|---|
| | Medium I* | Medium II | Medium III† | Medium IV | Medium V |
| Glucose | | 10 g. | 15 g. | | 50 g. |
| Mannitol | 50 g. | | | 15 g. | |
| Yeast extract | | 10 " | | | |
| Ammonium succinate | 8 " | | 980 mg. | 980 mg. | 8 " |
| $K_2HPO_4$ | | | 100 " | 100 " | 100 mg. |
| $KH_2PO_4$ | 1.0 " | | 700 " | 700 " | 700 " |
| $MgSO_4.7H_2O$ | 0.3 " | | 201 " | 201 " | 201 " |
| $CaCl_2.2H_2O$ | | | 5.95 " | 5.95 " | 5.95 " |
| $FeSO_4.7H_2O$ | | | 5.00 " | 5.00 " | 5.00 " |
| $ZnSO_4.7H_2O$ | | | 4.40 " | 4.40 " | 4.40 " |
| $MnSO_4.H_2O$ | | | 2.75 " | 2.75 " | 2.75 " |
| NaCl | | | 2.55 " | 2.55 " | 2.55 " |
| $CuCl_2.2H_2O$ | | | 0.269 " | 0.269 " | 0.269 " |
| $(NH_4)_6Mo_7O_{24}.4H_2O$ | | | 1.82 " | 1.82 " | 1.82 " |
| *i*-Inositol | | | 1.0 " | 1.0 " | 1.0 " |
| Thiamine HCl | | | 100 μg. | 100 μg. | 100 μg. |
| Pyridoxine HCl | | | 100 " | 100 " | 100 " |
| Calcium pantothenate | | | 100 " | 100 " | 100 " |
| Nicotinamide | | | 50 " | 50 " | 50 " |
| *p*-Aminobenzoic acid | | | 50 " | 50 " | 50 " |
| Biotin | | | 5 " | 5 " | 5 " |

*Reference 1. Final medium, in tap water, was adjusted to pH 5.2–5.4 with succinic acid. The other media were made up in distilled water.

†Reference 23.

trated at 40° C. under a stream of nitrogen to approximately 10 ml. This was shaken with 3 ml. of 0.1 *N* sulphuric acid and the aqueous layer separated. To it was added 6 ml. of Van Urk's reagent (*p*-dimethylaminobenzaldehyde (0.2 g.) and $FeCl_3.6H_2O$ (0.003 g.) in 100 ml. 65% (v/v) sulphuric acid). After one hour, the color of the sample was measured in a Coleman Junior Spectrophotometer at 550 m$\mu$ and the amount of alkaloid calculated as ergometrine was determined from a previously prepared standard curve.

*Recovery and Identification of Products*

Separation of the alkaloids was carried out by a modification of the method used by Stoll and Rüegger (21). The whole cultures were freeze-dried and shaken twice with 500 ml. petrol (b.p. 30°–60° C.) to remove the bulk of the fats, and the residue extracted once with 250 ml. of 2% tartaric acid in 70% aqueous acetone, followed by three further extractions of 100 ml. each. The extracts were concentrated at 40° C. *in vacuo* to remove the acetone, then extracted twice with 100 ml. of ether, and the extract discarded. The aqueous solution was neutralized with sodium carbonate, then extracted four times with 100 ml. of a mixture of chloroform and isopropanol (3 : 1). The extracts were concentrated *in vacuo* to a small volume (0.1 ml.), and a sample of the solution examined by circular paper chromatography. A disk of Whatman No. 1 filter paper (24 cm. diameter) was dipped in a mixture of equal volumes of formamide and ethanol (16) and well blotted. In order that the ethanol might evaporate, at least 15 min. was allowed to elapse after application of the alkaloid samples before the paper was placed in a large desiccator and irrigated with a solvent mixture consisting of four parts of benzene to one part of petrol (b.p. 100°–120° C.). The solvent front took approximately two hours to reach the outer edge of the paper. The air-dried chromatogram was examined under ultraviolet light of 2537 Å, and fluorescent zones noted. The paper was then sprayed with a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde dissolved in concentrated hydrochloric acid. Dark blue zones appeared on the chromatogram within a few minutes. A second sample of the culture extract was then chromatographed in the same manner using chloroform saturated with formamide as the mobile phase. Using the solvent mixture benzene–petrol (4 : 1) on formamide impregnated paper, it was found that a mixture of the following alkaloids could be separated, in order of increasing $R_f$ value: ergometrine, ergotamine, ergosine, ergotaminine, ergosinine, ergocornine, ergokryptine, ergocorninine, ergocristinine, and ergokryptinine. Ergocristine appeared between ergosinine and ergocornine, but since these two alkaloids themselves were very close together, inclusion of ergocristine in the mixture resulted in a single broad band in which the three individual components were indistinguishable. Ergosine and agroclavine were superimposed on ergotamine, while ergometrinine, elymoclavine, and penniclavine were superimposed on ergometrine in the solvent system. When the system chloroform–formamide was used, a mixture of penniclavine, ergometrine, ergometrinine, agroclavine, and ergotamine could be separated (in order of increasing $R_f$). Elymoclavine

was superimposed on ergometrine, while all the remaining alkaloids were superimposed on ergotamine at the solvent front in this system. Identification of the alkaloids in the culture samples was carried out by comparison of the zones with a mixture of standards in an adjacent segment of the chromatogram. Only those zones which gave a blue fluorescence in ultraviolet light as well as a blue or green zone with Van Urk's reagent were recorded, except with agroclavine and elymoclavine, which do not fluoresce.

## Results

When the three cultures were grown on medium I under stationary conditions and sampled after intervals of 25, 35, and 52 days, no alkaloid could be detected until the third sampling, when 0.51 to 1.5 mg. per liter of culture (calculated as ergometrine) were found in PRL 1555 and 1557. No alkaloid was found in PRL 1556. Duplicate experiments were carried out under conditions of dark and light and with added biotin (23), but no marked differences were noted. A positive color reaction, equivalent to 0.53 mg. of ergometrine per liter, was obtained at 35 days when shake cultures of PRL 1555 in medium I were used.

The influence of different media on production was tested by growing the three strains on the five media described and examining the cultures at 48 days. PRL 1555 alone was found to produce alkaloids in this experiment. PRL 1556 and 1557 gave no color reaction with Van Urk's reagent even when re-examined after 65 days. Medium I gave the best yield of alkaloid (0.55 mg. per liter culture) but some production also occurred on medium III (0.25 mg.) and II (the estimated value of 0.52 mg. obtained here was undoubtably too high owing to the presence of a brown pigment extracted along with the alkaloids). No appreciable production occurred on media IV and V. Paper chromatograms of extracts from five pooled flasks of PRL 1555 grown on medium I showed the presence of ergometrine and ergotamine.

TABLE II

ALKALOIDS FORMED IN STATIONARY CULTURES OF *Claviceps purpurea**

| PRL 1555 | PRL 1557 |
|---|---|
| Penniclavine | Ergometrine (and possibly elymoclavine) |
| Ergometrine (and possibly elymoclavine) | Agroclavine |
| Ergometrinine | Ergotamine |
| Agroclavine | Ergocornine |
| Ergotamine | Ergokryptine |
| Ergotaminine | Ergocorninine |
| Ergocornine | |
| Ergokryptine | |

*Fifty-day-old submerged culture on Abe's medium (1).

When PRL 1555 and 1557 were grown under stationary conditions for 50 days and the alkaloids from 20 pooled flasks of each were isolated and examined by paper chromatography, alkaloids were again detected (Table II).

## Discussion

The most notable differences in the conditions for alkaloid production used by Abe and his group, compared with those used by other workers, are the long incubation periods (30–50 days), the use of a medium consisting of 5% mannitol, 0.8% ammonium succinate, 0.1% monopotassium phosphate, 0.03% magnesium sulphate, and tap water, and the adjustment of the pH of the medium (with succinic acid) to pH 5.2–5.4. Under these conditions the formation of alkaloids by two of the three strains of *Claviceps* used was shown to take place and Abe's results confirmed. Although production at this level is of no commercial significance, it does serve to demonstrate that certain strains of *Claviceps* possess the ability to synthesize the lysergic acid molecule *in vitro*, and hence do not need to rely either on preformed nutrients from the host plant or formation of sclerotia. It seems reasonable to expect that further experiments may reveal conditions which will allow a significant increase in yield.

The work carried out up to the present indicates that three conditions are important. The first is selection of a proper strain. Of the three used above, one (PRL 1556) gave no production and a second (PRL 1557) gave only irregular production. Abe *et al.* (8) have also shown that strains vary in the types of alkaloid produced and it is of interest that the alkaloids detected in the *in vitro* cultures were also found in the sclerotia from which they were isolated. The composition of the medium is also important. Abe's medium was superior to all others tested, although it does not appear to be essential for production. Stoll *et al.* (22) also found that their culture would produce alkaloids on other media, and that production on Abe's medium could be improved by the addition of 5% sucrose. Finally, a long period of incubation is necessary. Our results indicate that at least 35 days are required for production when shake cultures are used and longer periods for stationary cultures. Stoll *et al.* (22) found 20 days were required for detectable production with shaken cultures and 42 days with stationary cultures.

The numerous reports of failure by other investigators to achieve alkaloid production is understandable since few, if any, of the above conditions were observed in their experiments.

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# STUDIES ON THE EFFECTS OF PHAGOCYTIC STIMULATION ON MICROBIAL DISEASE

## VII. A PRELIMINARY EXPERIMENTAL APPROACH TO AN UNDERSTANDING OF THE CORRELATION BETWEEN TUBERCULIN HYPERSENSITIVITY, THE FUNCTIONAL ACTIVITY OF THE RETICULOENDOTHELIAL SYSTEM, AND TUBERCULOUS LESIONS IN GUINEA PIGS[1]

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### Abstract

1,4-Dimethyl-7-isopropyl-bicyclo-decapentane (BD.I) has been shown to ameliorate the course of experimental tuberculosis of guinea pigs. Further experiments demonstrate that tuberculin reactions in BD.I treated animals, whether normal or immunized, were markedly milder than in similar but untreated groups of animals. Tuberculous lesions in BD.I treated normal and immunized guinea pigs were less severe when compared with untreated animals as seen at autopsy 6, 8, and 12 weeks after virulent superinfection. It seems that the drug does not interfere with the immune principle. Partial desensitization and the therapeutic effects are attributed to stimulation of the cellular defense apparatus.


### Introduction

1,4-Dimethyl-7-isopropyl-decapentane (BD.I) was found, in previous investigations, to induce a phagocytic activity of the endothelial cells of the small vessels in the skin of albino mice (11) and to increase the speed of ingestion of intravenously injected particles by the reticuloendothelial system if injected intramuscularly (17). *In vitro* and *in vivo* studies (13, 19) showed that BD.I stimulates the monocytes from a normal guinea pig to an increased phagocytic activity against BCG. Barton and Wendler (1) demonstrated that the phagocytic activity of leucocytes of the whole blood was increased against starch particles *in vitro* in the presence of BD.I It was concluded from these experiments and from a model experiment on the monocytes that BD.I stimulates the cellular defense mechanism of the organism, both locally and systemically. The beneficial action in experimental tuberculosis of BD.I alone (12, 18) or with an antituberculous agent (18) was attributed to its property of stimulating phagocytosis. Thus BD.I was described as a reticuloendothelial system stimulating agent (17), and several similarly acting substances, all oil soluble, were also described (14). In the present preliminary experiment, a further approach to an understanding of the mechanism of action of BD.I was attempted by trying to find the correlation between the fluctuations of the tuberculin hypersensitivity, the functional activity of the reticuloendothelial system, and the severity of the tuberculous disease in guinea pigs. The carefully controlled experiments of Rich (24), Rich and his coworkers (23, 25), Rotschild *et al.* (26), Birkhaug (3, 4), Raffel (21), and

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Suter (27) clearly demonstrated that hypersensitivity is independent of immunity and that the partial or complete abolition of the allergic status did not interfere with immunity but permitted it to operate to better advantage.

We approached the problem by attempting to desensitize tuberculin hypersensitive guinea pigs by intramuscular administration of BD.I as it was known that this compound was used successfully by several investigators for desensitizing allergic states both experimentally and clinically. Jancso (15) had claimed that chamazulene (naturally occurring dimethyl-isopropyl-bicyclo-decapentane) may desensitize the allergic organism against histamine. The same compound was used by Blazso (5, 6) to desensitize asthmatic newborn infants and children. Varga (29) treated allergic migraines with the dimethyl-isopropyl derivatives. Tuczek (28) attributed a desensitizing effect to BD.I in the treatment of bronchial asthma. He also observed that isonicotinic acid hydrazide treated tuberculous patients showed a negative tuberculin reaction after a period of treatment with BD.I. (Unfortunately, the author gave no details regarding this observation.) Gaspar, Németh, and Issekutz (10) obtained "an endogen histamine desensibilization" for albino rats, by appropriate treatment with the dimethyl-isopropyl derivative. Although the tuberculin type hypersensitivity (delayed) and the anaphylactic hypersensitivity (immediate type) differ in manifestations, both are of basically similar characteristics (22), and further information on the mechanism of action of BD.I was sought for by a correlation of an interfering with the allergic status and the above described action of BD.I on cells belonging to the reticuloendothelial system.

## Materials and Methods

Ninety-six male guinea pigs, giving a negative skin reaction to 10 mg. of old tuberculin, were divided into four groups, each comprising 24 animals. Each group contained six albino animals and the remaining 18 animals were of mixed colors but with white abdominal areas so as to permit easy and correct readings of the tuberculin test. The use of animals of mixed colors was preferred in order to avoid using the somewhat more resistant albino guinea pigs. All animals were obtained at the same time from the same supply house. Each group was divided into three subgroups carefully selected as to color so that each subgroup of eight animals contained two albino animals and one representative of all the other colors. Group I served as normal controls. Group II were the normal desensitized. Animals of Group III were the immune allergic and those of Group IV the immune desensitized. All the animals weighed approximately 450 g. at the day on which the experiment was started. The animals were kept in groups of eight in separate metal cages and were under observation for 14 days prior to the experiment. A further 20 tuberculin-negative animals with the same characteristics were divided into the same four groups and kept separately. These reserve groups were treated in exactly the same manner as the four main groups participating in the experiment itself. The purpose of these reserve groups was to ensure

having 24 animals in each group on the day superinfection occurred by immediate replacement of animals of the main groups which might die.

The course of the experiment is presented schematically in Figs. 1 and 2. On zero day, the animals of the immune allergic and immune desensitized groups were inoculated intradermally with 10 mg. moist weight of BCG in 0.2 ml. of Sauton medium (diluted 1 : 4) at the lower left side of the depilated abdominal wall. The immunizing antigen was kindly supplied by the Institute of Microbiology and Hygiene of the University of Montreal. The BCG strain was grown on Sauton medium and harvested on the 14th day. The homogenized milky suspension contained microscopic clumps. The inoculating suspension was used on the day on which it was gathered from the medium. Only 0.2 ml. of the inoculating suspension was taken into the syringe at any one time—sufficient for the injection of one animal. Sedimentation was avoided by repeated forcing of the BCG suspension in and out of the syringe before every inoculation. Normal control and normal desensitized animals were inoculated at the same time and in the same way with 0.2 ml. of Sauton medium, diluted 1 : 4.

Tuberculin tests were performed systematically and periodically on all the groups, including the normal control and normal desensitized animals. The first test was performed on the 20th day after the sensitizing inoculations, the second test 16 days after the first, and the following test approximately every 4th week thereafter. In every case the guinea pigs were tested intradermally with 5 mg. of old tuberculin in 0.1 ml. saline solution. Readings were made at 48 hours to avoid disturbing non-specific erythematous reactions. The approximate volume of erythema and induration was registered in $mm.^3$ obtained by multiplying the maximum diameter by the minimum diameter by half of the thickness of the folded skin. The area of central necrosis was measured by multiplying the maximum diameter by the minimum diameter. Animals of the non-immunized groups received the same intradermal tuberculin injection as did the two other groups, partly to reveal any accidental formation of hypersensitivity in the normal groups and partly to avoid any action other than provoking tuberculin reactions in the hypersensitive animals, brought about by monthly intradermal injections of 5 mg. of old tuberculin.

After a satisfactory state of tuberculin hypersensitivity was established in the immunized groups, the process of desensitization was started on the 22nd day after vaccination. For the normal desensitized and the immune desensitized groups 10 mg. of BD.I in 0.3 ml. linseed oil was injected intramuscularly for each guinea pig once weekly during the whole period of the experiment, till all animals were killed. Animals of the normal control and immune allergic groups received 0.3 ml. of linseed oil intramuscularly, every week for the same period of time. Since a partial desensitization against 5 mg. of old tuberculin was obtained in the two BD.I treated groups, all the animals of the four groups were superinfected 20 weeks after the intradermal vaccination with BCG. The animals were injected subcutaneously in the right groin with 0.1 mg. (moist weight) of *M. tuberculosis* var. *bovis* (Ravenel strain) in 0.5 ml. phosphate buffered solution. The suspension was obtained

from a 7-day-old culture in Dubos liquid medium. Previous experiments showed that the above doses would ensure the death of all the control animals within 120 days.

Desensitization and tuberculin tests were continued, as stated above, for the period following superinfection. The need for this was indicated by the observations of Carlinfanti (8) and Birkhaug (2) that animals regained tuberculin hypersensitivity in about 10 to 14 days after the last desensitizing dose. At the end of the 6th week after superinfection, seven to eight animals of each group were killed. Similarly eight animals were killed out of each group at the end of the 8th and 12th week after superinfection. Animals which had died not more than 10 days before others of the same group were killed, were considered in the evaluation as belonging to the group of the eight killed animals. The autopsy was performed as soon as possible after death occurred or the animal was killed. The carefully dissected glands and organs were examined and visible tuberculous lesions registered by the method described by Karlson and Feldman (16). All the animals were weighed once a week and the average body weight was plotted against time in weeks on millimetric scale paper as presented in Figs. 1 and 2.

## Results

The intracutaneous method of vaccination is considered by Birkhaug (2) to be the most suitable method of rendering guinea pigs both immune and allergic and observations in our laboratories confirm this. The present experiment confirms this observation. Four to 6 days after the BCG inoculation the usual inflammatory process developed at the site of the intradermal injection. In a few days a central necrosis and skin perforation occurred, followed by a dense discharge containing a great number of acid-fast bacilli. In 15 to 23 days, the lesions showed signs of healing and by the end of the 4th week all lesions were completely healed. During the first 3 weeks, the general appearance of BCG inoculated animals was somewhat weaker than that of control animals. Immunized animals either lost weight (15 to 30 g.) or maintained their original weight for the first 2 weeks. The weight curves became normal from the 15th day as seen in Fig. 1. The first tuberculin test performed 20 days after vaccination revealed that all the animals in the immune desensitized groups showed positive tuberculin reactions. The average volume of erythma and induration was 323 mm.$^3$ and 423 mm.$^3$ respectively. Six animals in both groups showed a central necrosis. This state of tuberculin hypersensitivity was considered to be satisfactory for starting the desensibilization process on the 22nd day after vaccination. The animals of both the normal desensitized and immune desensitized groups tolerated well the weekly injections of 10 mg. BD.I. No localized redness or swelling, no lassitude or anorexia was observed following the administration of BD.I. On the contrary, as previously described (12), both groups treated with BD.I increased in weight as compared with the non-treated groups, and as can be seen from the average body weight curves in Figs. 1 and 2.

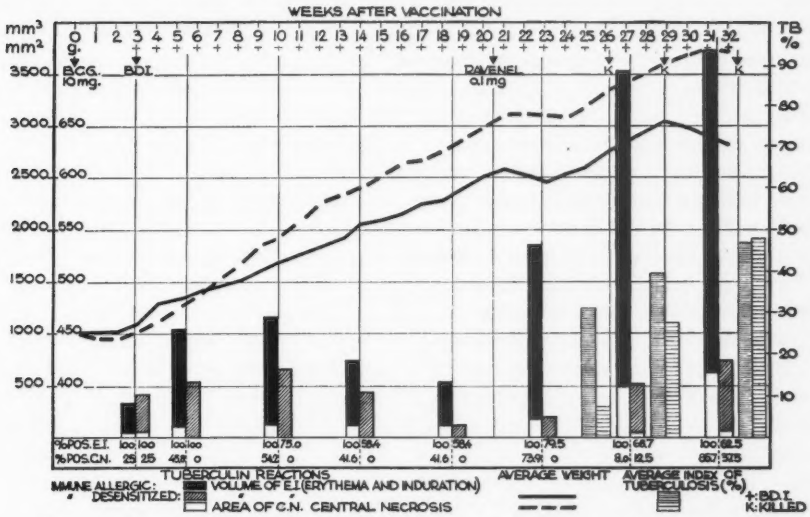


FIG. 1. Tuberculin reactions, the average weight, and the average index of tuberculosis of immune allergic and immune desensitized guinea pigs. Superinfection took place at the 20th week after immunization (BCG). BD.I administered at the time marked +.

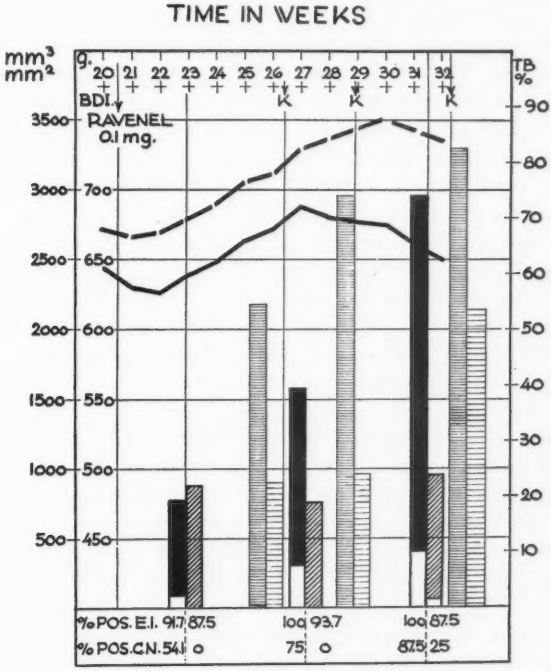


FIG. 2. Tuberculin reactions, the average weight, and the average index of tuberculosis of normal-allergic and normal-desensitized guinea pigs. Assignment as in FIG. 1.

None of the guinea pigs in the normal control and normal desensitized groups became tuberculin hypersensitive during the time from the vaccination to the virulent inoculation. During this period, tuberculin reactions performed at 4 week intervals, 100% of immune allergic animals remained hypersenstive to 5 mg. of old tuberculin, showing an average erythema and induration of 1037 $mm.^3$ at the 5th week, 1159 $mm.^3$ at the 10th week, 729 $mm.^3$ at the 14th week, and 541 $mm.^3$ at the 18th week after vaccination. In every instance nearly half the animals showed central necrosis in the tuberculin reactions. The area of central necrosis was relatively small in the immune allergic group during the postvaccination period and was quite constant, varying between 92 $mm.^2$ and 120 $mm.^2$ as shown in Fig. 1. Tuberculin reactions taken at the 5th week with 5 mg. of old tuberculin for animals of the immune desensitized groups showed no central necrosis but an average erythema and induration volume of 520 $mm.^3$ However, all of the animals in this group reacted positively. At the 10th week after BCG vaccination, 75% of this group reacted positively with an average erythema and induration volume of 660 $mm.^3$, at the 14th week 58.4% with an average volume of 445 $mm.^3$, and at the 18th week the same percentage reacted positively with an average erythema and induration volume of 104 $mm.^3$ In the immune desensitized group during the postvaccination period and after the desensitization had begun, there was no central necrosis registered on the skin following tuberculin testing.

Figs. 1 and 2 show that when superinfection was given at 21 weeks after BCG inoculation, nearly half of the immune desensitized animals showed no tuberculin hypersensitivity to 5 mg. of old tuberculin injected intradermally, and none of the reacting animals showed central necrosis. At this same time, all the immune allergic animals reacted positively, 41.6% of them with central necrosis. The immune desensitized animals had an average volume of erythema and induration of 104 $mm.^3$ and the immune allergic animals an average of erythma and induration of 541 $mm.^3$

On the day of virulent superinfection, 23 guinea pigs in the immune allergic group and the 24 animals in each of the three other groups were injected with 0.1 mg. moist weight of the Ravenel strain. Three of the control animals, two of the normal desensitized group, two of the immune desensitized group, and one of the immune allergic group died during the postvaccination period and were replaced from among the reserve animals. Care was taken to ensure that the replacements were, as nearly as possible, of the same weight and same general hypersensitivity as the animals replaced. The one animal of the immune allergic group that died could not be replaced (there was no suitable replacement). One animal from the immune desensitized group died 3 days after the virulent inoculation and was excluded from the experiment. Another animal from the same group died during the 7th week and one during the 8th week after superinfection. Both showed severe tuberculous involvement of the organs. These animals were included for evaluation purposes in the group sacrificed at the 8th week. One other guinea pig from the normal control animals died during the 9th week and was included in the last group of eight sacrificed.

After a slight loss of weight during the first 2 weeks following the virulent superinfection, all animals showed gains in weight. For the whole postinfection period, the two groups treated with BD.I appeared to be more active and healthier than the groups not so treated. The weight curves for the four groups, presented in Figs. 1 and 2 show the curves of the desensitized groups to be constantly higher than those of the allergic groups. Normal control animals began to lose weight 6 weeks after infection with the Ravenel strain of *Bacillus tuberculosis*, normal desensitized animals after 9 weeks. The immune allergic and the immune desensitized groups started to lose weight 8 weeks and 11 weeks respectively after superinfection.

At the 2nd, 6th, and 10th weeks after superinfection, all the animals were tested with 5 mg. of old tuberculin. The readings, made at 48 hours, are presented in Table I and Figs. 1 and 2 respectively. We noted, at first, that the highest volume of erythema and induration is observed in the immune allergic group and markedly less severe reactions were encountered in the normal control animals. However, 91–100% of the animals in both groups reacted positively. In the normal desensitized group, nearly all the animals reacted positively but the volume of erythema and induration was markedly weaker than in the control group. More animals were desensitized in the immune allergic group but the degree of desensitization was approximately the same as that of the normal desensitized group. Thus only a weak desensitization could be claimed from the resulting average volume of erythema and induration but the distribution of this percentage change and the area of central necrosis observed are very instructive. While a large percentage of the animals in the control and immune allergic groups showed positive central necrosis, none of the normal desensitized animals showed central necrosis during the 6 weeks after the virulent infection and only a weak central

TABLE I

TUBERCULIN REACTIONS IN NORMAL AND IMMUNE GUINEA PIGS AT DIFFERENT TIME INTERVALS AFTER INFECTION. AVERAGE VOLUME OF ERYTHEMA AND INDURATION AND CENTRAL NECROSIS IN ALLERGIC AND DESENSITIZED GROUPS

| Groups | No. animals | Week after infection | % pos. E.I. | Av. vol. E.I., mm.$^3$ | % pos. C.N. | Av. area C.N., mm.$^2$ |
|---|---|---|---|---|---|---|
| Normal controls | 24 | 2 | 91.7 | 790 | 54.1 | 95 |
| | 16 | 6 | 100 | 1587 | 75 | 292 |
| | 8 | 10 | 100 | 2944 | 87.5 | 391 |
| Normal desensit. | 24 | 2 | 87.5 | 913 | 0 | 0 |
| | 16 | 6 | 93.7 | 762 | 0 | 0 |
| | 8 | 10 | 87.5 | 979 | 25 | 68 |
| Immune allergic | 23 | 2 | 100 | 1852 | 73.9 | 179 |
| | 15 | 6 | 100 | 3525 | 80 | 504 |
| | 7 | 10 | 100 | 3773 | 85.7 | 608 |
| Immune desensit. | 23 | 2 | 79.5 | 195 | 0 | 0 |
| | 16 | 6 | 68.7 | 515 | 12.5 | 41 |
| | 8 | 10.9 | 62.5 | 782 | 37.5 | 66 |

necrosis was observed for 12.5% of the immune desensitized animals at the 6th week after infection. In the desensitized groups, normal and immune, the central necrosis increased in percentage and area at the 10th week after the Ravenel infection, as at the same time fatal tuberculous lesions developed in the organs and lymph nodes. Even at this stage, the area of central necrosis in the desensitized groups was not over 20% of the average necrotic area of the control and immune allergic groups. An examination of Figs. 1 and 2 shows also that the allergizing principle of the Ravenel strain is higher than that of the BCG strain, and that guinea pigs rendered hypersensitive with the BCG strain become more hypersensitive after the superinfection than the normal control animals.

The results at autopsy are presented in Figs. 1 and 2 and in Table II. Tuberculous lesions in lungs, liver, spleen, and lymph nodes of the four groups at the 6th, 8th, and 12th weeks after virulent infection are compared using visible lesions as the index of disease. Each organ was given a rating of 0 to 4 according to the amount of gross tuberculous disease. Maximum total therefore for each animal was 16 (100%). In the normal as well as immune groups the desensitized animals showed markedly less tuberculous involvement than the allergic groups at the 6th and 8th weeks. At the 8th week there was no difference between amount of involvement in the normal desensitized and immune desensitized groups. At this time normal desensitized animals showed markedly fewer visible lesions than the normal controls, but immune allergic and immune desensitized animals showed tuberculous disease of equal involvement.

TABLE II

AVERAGE INDEX OF TUBERCULOSIS IN NORMAL AND IMMUNIZED GUINEA PIGS AT DIFFERENT TIMES AFTER INFECTION. THE INDEX OF INVOLVEMENT IN ALLERGIC AND DESENSITIZED GROUPS

| Groups | No. animals | Killed weeks after infection | Av. index of tuberculosis | | | | | % T.B. |
|---|---|---|---|---|---|---|---|---|
| | | | Lungs | Liver | Spleen | Lymph nodes | Totals | |
| Normal controls | 8 | 6 | 2.2 | 2.1 | 1.8 | 2.6 | 8.7 | 54.4 |
| | 8 | 8 | 2.4 | 2.0 | 2.8 | 3.6 | 10.8 | 67.5 |
| | 8 | 10 | 3.2 | 3.4 | 2.8 | 3.8 | 13.2 | 87.5 |
| Normal desensit. | 8 | 6 | 0.8 | 0.6 | 0.6 | 1.6 | 3.6 | 22.5 |
| | 8 | 8 | 1.0 | 1.3 | 2.4 | 2.7 | 7.4 | 46.2 |
| | 8 | 10 | 1.4 | 1.4 | 2.7 | 2.9 | 8.5 | 53.1 |
| Immune allergic | 8 | 6 | 1.0 | 0.8 | 1.2 | 2.0 | 5.0 | 31.5 |
| | 8 | 8 | 1.2 | 0.9 | 2.1 | 2.0 | 6.2 | 38.7 |
| | 7 | 10 | 2.6 | 1.1 | 1.1 | 2.7 | 7.5 | 46.8 |
| Immune desensit. | 7 | 6 | 0 | 0 | 0.3 | 0.9 | 1.2 | 7.5 |
| | 8 | 8 | 0.9 | 0.8 | 1.1 | 1.6 | 4.4 | 27.5 |
| | 8 | 10 | 2.4 | 1.4 | 1.4 | 2.4 | 7.6 | 47.6 |

## Discussion

Tuberculosis of normal and BCG-immunized guinea pigs was influenced beneficially by treatment with BD.I; the tuberculous lesions were less severe in character and the tuberculin reactions appeared to be milder in comparison with normal and immunized untreated groups. If the present experiments are compared with observations of those investigators (24, 26, 27, 3, 4) who desensitized tuberculin hypersensitive guinea pigs with subsequent administration of tuberculin, we may confirm that a diminished state of hypersensitivity does not lower the immunity but even permits the immune principle to function better. Thus, the administration of a simple non-protein compound, like BD.I, may partially or completely prevent the guinea pigs from developing tuberculin hypersensitivity. Desensitization with tuberculin is followed by toxic manifestations, such as loss of weight, reduced muscle tone, persistent anorexia, and lassitude, or by excessive mortality. The same manifestations were not observed following BD.I administration. On the contrary, BD.I treated animals showed consistently higher average body weight than animals of the allergic groups. This difference could be explained by the fact that BD.I is not a protein and does not interfere with the state of hypersensitivity.

Rich and Lewis (25) added tuberculin to surviving tissues of hypersensitive animals and observed severe cellular destruction of the tissues. Intracutaneous or subcutaneous tuberculin administration induces an inflammatory process with local and systemic response which is connected with the antigen characteristics of the tuberculin and with the state of hypersensitivity. Inflammatory processes due to subsequent administration of tuberculin into the hypersensitive organism may lead to the formation of the same mediators, described by Menkin (20) and others, that are formed in any inflammatory process. The mediators, influencing beneficially the outcome of an inflammation, act on cells belonging to the reticuloendothelial system. Since these mediators stimulate the reticuloendothelial system to an increased activity in defense, it is obvious to suppose that, as in the experiments of Rich (24), Rotschild *et al.* (26), and Birkhaug (3, 4), subsequent tuberculin administration influences beneficially the outcome of the tuberculous disease by its indirect action on the reticuloendothelial system.

If we accept as proved that BD.I stimulates the cellular defense mechanism of the host to an increased activity, the negative or mild tuberculin reactions of the BD.I treated animals, with no necrotic characteristics, reflect a better functioning of the reticuloendothelial system in the desensitized animals than in the allergic groups. BD.I treatment does not interfere with the immunity of an animal since no quantitative differences existed between the index of tuberculosis and degree of desensitization of BD.I treated groups, whether immune or normal.

The important role of the reticuloendothelial system in the tuberculin reactions was also reflected in the data of Bluefarb (7), who demonstrated that tuberculous or BCG immunized individuals suffering from diseases of the reticuloendothelial system reacted with negative or mild tuberculin reactions.

This, according to Bluefarb, is due to the damaged function of the reticuloendothelial system. This observation does not contradict our own. Tuberculin reactions may fail with individuals with an impaired reticuloendothelial system. In these cases, the tuberculous disease may be expected to be fulminant. On the other hand, the tuberculin reactions may be mild and show absence of necrosis in animals where there is stimulation of the reticuloendothelial function and where the defense apparatus is functioning to better advantage. Under these circumstances the animal is able to prevent, completely or partially, the development of erythema and induration and central necrosis, which are unnecessary and harmful inflammatory responses to tuberculin administration. In this later case, the outcome of the disease is influenced beneficially. So the state of hypersensitivity can camouflage the immune principle and can be considered as a defect in the function of the reticuloendothelial system.

Recently, Cornforth *et al.* (9) reported beneficial action of Triton WR1339 on experimental tuberculosis. The mechanism of action was attributed to its stimulating action on the monocytes. Depression of tuberculin sensitivity in the guinea pig was observed, following the treatment with Triton WR1339. This observation similarly supports our above discussed view concerning the relation between hypersensitivity and functional stimulation of the cellular defence mechanism.

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# THE RELATIONSHIP BETWEEN SOIL BACTERIA WITH SIMPLE NUTRITIONAL REQUIREMENTS AND THOSE REQUIRING AMINO ACIDS[1]

T. M. B. Payne, J. W. Rouatt, and A. G. Lochhead

## Abstract

Twenty-two of 30 representative soil bacteria having simple nutritional requirements, in that they showed maximum development in a medium of inorganic salts and sugar, were found to be able to synthesize a variety of amino acids, though in no single case were more than four ninhydrin-positive substances found. The culture filtrates permitted the growth of amino-acid-requiring bacteria to the extent that they contained the specific amino acid or acids required by strains of the latter group. The findings point to an associative action between these nutritional groups of soil organisms and suggest that the preferential stimulation in the rhizosphere of bacteria requiring amino acids may be ascribed in part to the ability of the other group of bacteria, which are numerically increased in the rhizosphere, to synthesize amino acids.

## Introduction

Comparative studies of the relative incidence of soil bacteria of different nutritional requirements have demonstrated that one of the most characteristic rhizosphere effects is the preferential stimulation of organisms requiring amino acids for maximum growth (6). Soil bacteria with the simplest nutritional requirements, namely, the ability to develop maximally in a glucose – nitrate – mineral salts medium, are also normally stimulated in the rhizosphere (6, 7, 10). Lochhead and Thexton (7) studied the associative effect of this nutritional group on the growth of bacteria requiring amino acids and reported that cell-free filtrates of the basal medium, in which bacteria with the simplest nutritional requirements had been grown, were capable of supplying the nutrients necessary for the growth of a large percentage of the amino acid requiring bacteria tested.

A number of workers have subsequently reported the occurrence of free amino acids in the medium in which various bacteria had grown. Dunlop (3) found valine, leucine, and methionine extracellularly in culture filtrates of *Escherichia coli*. Histidine, alanine, and aspartic and glutamic acid have also been reported in filtrates of *E. coli* or *Aerobacter aerogenes* (2). Woiwod and Proom (13) stated that *Clostridium tetani* and *Proteus vulgaris* produced polypeptides whereas *Serratia marcescens* produced arginine in the culture medium and they considered the possibility of these substances being synthesized and then excreted by the organisms.

The results reported by Lochhead and Thexton (7) and the apparent ability of some bacteria to excrete amino acids suggest that soil organisms with the simplest nutritional requirements may contribute to the total free amino acid content of soil and thus be a factor in the stimulation of amino acid requiring bacteria in the rhizosphere of growing plants.

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## Experimental

### Cultural Studies

*Cultures*

A number of cultures of soil bacteria of known nutritional requirements, which had been isolated from the rhizospheres of a variety of crops and control soils, were used. Thirty cultures, which after reisolation from soil extract agar plates and repeated subculturing demonstrated the ability to develop maximally in the basal medium, were selected as being representative of the group with simple nutritional requirements and designated as 'B' group organisms. Similarly 30 bacterial isolates which failed to develop in the basal medium but showed maximum growth in the presence of vitamin-free 'Casamino acids' were selected as representative of the amino acid requiring group and designated as 'A' group organisms. These stock cultures were maintained in soil extract semisolid agar.

*Media and Methods*

The media employed in the studies were as follows:

*Medium B*—Basal medium of glucose – nitrate – mineral salts as described by Lochhead and Chase (5).

*Medium A*—Medium B + 0.4% vitamin-free 'Casamino acids'.

*Medium G*—Medium B + growth factors (5).

*Soil extract agar* and *soil extract semisolid agar* (5).

The individual amino acid requirements of the 'A' group organisms were determined by their growth response in Medium B + 0.025% of the following amino acids tested singly:

*dl*-α-Alanine
*l*-Arginine hydrochloride
*dl*-Asparagine
*dl*-Aspartic acid
*l*-Cysteine hydrochloride
Diiodo-*l*-tyrosine
*l*-Glutamic acid
Glycine
*l*-Histidine hydrochloride
*l*-Hydroxyproline
*dl*-Isoleucine
*l*-Leucine
*dl*-Lysine hydrochloride
*dl*-Methionine
*dl*-Phenylalanine
*l*-Proline
*dl*-Serine
*dl*-Threonine
*l*-Tryptophan
*l*-Tyrosine
*dl*-Valine
*l*-Taurine
*dl*-Norleucine

The amino acids were sterilized by passage through sintered glass filters and added aseptically to tubes of Medium B.

The 'B' group organisms were grown in flasks of basal medium with continuous agitation on a shaking machine. The effect of their cell-free filtrates on the growth of 'A' organisms was determined by adding aseptically 1 ml. of a sintered-glass-sterilized filtrate to 4 ml. of Medium B in tubes.

Tubes of each amino acid medium, each cell-free filtrate medium, and control media were inoculated with one loopful of a suspension of each of the 30 different strains of 'A' group organisms. Each series was run in duplicate and repeated.

The pH of the media was adjusted to 6.6–6.8 and unless otherwise stated all cultures were incubated for 5 days at 26° C. before the growth response was read or the amino acid content of the filtrates determined.

## Chromatographic Studies

### *Concentration of Filtrates*

Cultures of the 'B' group organisms were grown in 100 ml. of Medium B or G. Cell-free filtrates were obtained from these cultures by centrifugation and passage of the supernatant through sintered glass filters. The filtrates were concentrated by freeze-drying and the resulting residues extracted with 1 or 2 ml. of 80% ethanol.

### *Partition Chromatography*

The ethanol solutions of the concentrated culture filtrates were examined by ascending chromatography on sheets of Whatman No. 1 or No. 4 paper ($22\frac{1}{2} \times 18\frac{1}{2}$ in.). The concentrates were applied to the paper with a calibrated platinum loop until the equivalent of about 10 ml. of the original medium had been spotted. Each loopful was allowed to dry before further application. One-dimensional chromatograms were run with either 75% ethanol or *n*-butanol + acetic acid (12). Two-dimensional chromatograms were run with *n*-butanol + acetic acid and phenol (12) as the second solvent. The chromatograms were kept overnight (16 hours) at a temperature of 22° ± 2° C. After air-drying in a fume cabinet the papers were sprayed with 0.2% ninhydrin in 95% ethanol and developed in darkness for 48 hours at room temperature or dipped in 0.2% isatin in acetone containing 4% acetic acid (9) and developed for 10 minutes at 100° C.

### *Filter Paper Electrophoresis*

Filter paper electrophoresis was employed to separate some of the mixtures of amino acids which tended to run together with paper partition chromatography. The apparatus and method used were essentially similar to those employed by McKinley *et al.* (8) but with potassium acid phthalate + sodium hydroxide buffer (pH 5.9) or 5 *N* acetic acid buffer (pH 1.7) as described by Durrum (4) as electrolytes. Sørensen's phosphate buffer (pH 8.0) (1) and McIlvaine's phosphate – citric acid buffer (pH 3.6) (1) at 0.05 ionic strength were also used as electrolytes. A current of 1 ma. per inch width of paper was applied for periods of 6 to 12 hours. The dried strips were developed with ninhydrin or isatin.

### *Intracellular Amino Acid Content of Bacteria*

In the preparation of a number of 'B' group filtrates for chromatography the cells removed from the medium by centrifugation were washed with four

successive washings of physiological saline and resuspended in 10 ml. of fresh basal medium. The cellular suspension was then subjected to 5 minutes sonic vibration (Raytheon). The cellular debris was removed by passage through a sintered glass filter and the filtrate immediately concentrated by freeze-drying. The residue was taken up in 80% ethanol and applied to paper for chromatography in a quantity comparable to that of the concentrated medium from which the cells had been removed.

## Results

### *Growth Response of Amino-acid-requiring Bacteria*

Table I shows the growth response of the amino-acid-requiring bacteria to cell-free filtrates of 'B' group bacteria and the total number of individual amino acids utilized. All of the 'B' group filtrates were capable of supporting the growth of a number of 'A' group organisms. The 'A' group bacteria showed considerable diversity both in their ability to utilize 'B' group filtrates and individual amino acids. The nature of this variation is shown in Tables II and III by the comparison of the growth response of 10 representative amino-acid-requiring bacteria to 10 representative 'B' group filtrates and to

TABLE I

ABILITY OF CELL-FREE FILTRATES OF 'B' GROUP BACTERIA TO SUPPORT GROWTH OF 'A' GROUP BACTERIA AND THE GROWTH RESPONSE OF 'A' GROUP BACTERIA TO THE FILTRATES AND INDIVIDUAL AMINO ACIDS

| Effect of 'B' group filtrates | | | | Response of 'A' group organisms | | | |
|---|---|---|---|---|---|---|---|
| | | 'A' group bacteria | | | 'B' group filtrates | | |
| 'B' group filtrates (Cult. No.) | | No. of strains tested | No. able to grow in Med. B + filtrate | 'A' group bacteria (Cult. No.) | No. of filtrates tested | No. that supported growth | No. of single amino acids supporting growth |
| A | 24 | 30 | 14 | A 53 | 30 | 21 | 13 |
| AR | 49 | 30 | 10 | OS 29 | 30 | 2 | 2 |
| AR | 59 | 30 | 14 | OS 36 | 30 | 0 | 0 |
| AR | 65 | 30 | 16 | OS 48 | 30 | 0 | 0 |
| F | 32 | 30 | 17 | OS 59 | 30 | 5 | 0 |
| F | 46 | 30 | 22 | OS 65 | 30 | 10 | 0 |
| F | 56 | 30 | 19 | OS 67 | 30 | 29 | 4 |
| FR | 19 | 30 | 8 | OS 69 | 30 | 0 | 0 |
| FR | 79 | 30 | 13 | OR 38 | 30 | 3 | 0 |
| FR | 103 | 30 | 13 | OR 82 | 30 | 10 | 0 |
| OS | 9 | 30 | 8 | RCS 30 | 30 | 26 | 15 |
| OR | 4 | 30 | 6 | RCS 61 | 30 | 28 | 11 |
| OR | 52 | 30 | 11 | T 32 | 30 | 6 | 2 |
| RCS | 15 | 30 | 8 | T 39 | 30 | 26 | 15 |
| RCS | 64 | 30 | 10 | T 42 | 30 | 23 | 1 |
| SWD | 6 | 30 | 4 | T 52 | 30 | 7 | 0 |
| SWD | 17 | 30 | 14 | T 74 | 30 | 24 | 6 |
| SWD | 32 | 30 | 11 | T 82 | 30 | 7 | 0 |
| T | 23 | 30 | 13 | T 89 | 30 | 5 | 0 |
| T | 24 | 30 | 12 | T 117 | 30 | 4 | 0 |
| T | 28 | 30 | 19 | TR 19 | 30 | 20 | 3 |
| T | 38 | 30 | 17 | W 16 | 30 | 5 | 0 |
| T | 104 | 30 | 13 | W 21 | 30 | 27 | 11 |
| TR | 4 | 30 | 9 | W 33 | 30 | 13 | 0 |
| TR | 8 | 30 | 8 | W 65 | 30 | 3 | 0 |
| TR | 24 | 30 | 5 | W 99 | 30 | 17 | 9 |
| TR | 27 | 30 | 16 | W 103 | 30 | 1 | 0 |
| TR | 35 | 30 | 8 | WR 10 | 30 | 27 | 19 |
| TR | 36 | 30 | 12 | WR 95 | 30 | 3 | 5 |
| WR | 89 | 30 | 15 | WR 105 | 30 | 13 | 1 |

## TABLE II

### GROWTH RESPONSE OF "A" GROUP BACTERIA TO CELL-FREE FILTRATES OF "B" GROUP BACTERIA

| Filtrate of "B" group bacteria (Cult. No.) | "A" group bacteria (Cult. No.) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | OS 29 | OS 59 | OR 82 | RCS 30 | RCS 61 | T 32 | T 42 | TR 19 | W 21 | WR 10 |
| F 46 | – | X | – | X | X | X | – | X | X | X |
| FR 103 | – | – | X | X | X | – | X | – | X | X |
| OS 9 | – | – | – | X | X | – | – | – | X | – |
| OR 52 | – | – | – | X | X | – | – | – | X | X |
| RCS 64 | – | – | – | X | X | – | X | – | X | X |
| SWD 6 | – | – | – | X | – | – | – | – | – | X |
| T 24 | – | – | X | X | X | – | X | X | – | X |
| T 28 | – | – | – | X | X | X | X | X | X | X |
| T 38 | – | – | – | X | X | – | X | X | X | X |
| TR 24 | – | – | – | X | X | – | – | X | – | X |
| Controls | | | | | | | | | | |
| Med. B | – | – | – | – | – | – | – | – | – | – |
| Med. A | X | X | X | X | X | X | X | X | X | X |

NOTE: X = growth; – = no growth.

## TABLE III

### GROWTH RESPONSE OF "A" GROUP BACTERIA TO INDIVIDUAL AMINO ACIDS

| Amino acid | Total No. "A" group bacteria able to utilize | "A" group bacteria (Cult. No.) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | OS 29 | OS 59 | OR 82 | RCS 30 | RCS 61 | T 32 | T 42 | TR 19 | W 21 | WR 10 |
| Alanine | 5 | – | – | – | X | – | – | – | – | X | X |
| Arginine | 5 | – | – | – | – | – | – | – | X | X | X |
| Asparagine | 5 | – | – | – | X | X | – | – | X | – | X |
| Aspartic acid | 4 | – | – | – | X | X | – | – | – | X | X |
| Cysteine | 2 | – | – | – | – | – | – | – | – | – | X |
| Diiodotyrosine | 2 | – | – | – | X | – | – | – | – | X | – |
| Glutamic acid | 3 | – | – | – | – | – | – | – | – | X | X |
| Glycine | 3 | – | – | – | X | – | – | – | – | – | X |
| Histidine | 7 | X | – | – | – | X | – | – | – | X | X |
| Hydroxyproline | 6 | – | – | – | – | – | – | – | – | X | X |
| Isoleucine | 2 | – | – | – | X | – | – | – | – | – | – |
| Leucine | 7 | – | – | – | X | X | – | – | – | – | X |
| Lysine | 6 | – | – | – | X | X | – | – | – | X | X |
| Methionine | 12 | – | – | – | X | X | – | X | – | X | X |
| Norleucine | 4 | – | – | – | X | – | – | – | – | – | – |
| Phenylalanine | 4 | – | – | – | – | X | – | – | – | – | X |
| Proline | 5 | – | – | – | X | – | – | – | X | – | X |
| Serine | 4 | – | – | – | X | X | – | – | – | – | X |
| Taurine | 7 | – | – | – | – | X | X | – | – | X | X |
| Threonine | 7 | – | – | – | X | X | – | – | – | – | X |
| Tryptophan | 4 | – | – | – | X | – | X | – | – | – | – |
| Tyrosine | 10 | X | – | – | X | X | – | – | – | X | X |
| Valine | 3 | – | – | – | – | – | – | – | – | – | X |
| Controls | | | | | | | | | | | |
| Med. B | 0 | – | – | – | – | – | – | – | – | – | – |
| Med. A | 30 | X | X | X | X | X | X | X | X | X | X |

NOTE: X = growth; – = no growth.

individual amino acids. As indicated by these tables, of all the 'A' group organisms tested no two showed similar nutritional requirements either in respect to their ability to grow in 'B' group filtrates or in individual amino acids.

*Occurrence of Free Amino Acids in 'B' Group Filtrates*

It was possible by chromatographic techniques to demonstrate the occurrence of ninhydrin-positive substances in 22 of the 30 cell-free filtrates of the basal medium in which 'B' group bacteria had been grown. It was found that in most cases the production of ninhydrin-positive substances was enhanced by the inclusion of growth factors in the medium. The presence in the concentrated filtrates of salts, glucose, and substances of bacterial origin tended to produce considerable tailing and affected the $R_f$ values of the ninhydrin-positive substances, as could be readily demonstrated by the addition of a known amino acid to the concentrated filtrate. These factors, combined with the small quantities of free amino acids present, precluded the positive identification of a number of the ninhydrin-positive spots. It was, however, possible in many cases to identify the substances found by a combination of methods. The presence of alanine, aspartic acid, glutamic acid, glycine, lysine, methionine, proline, tryptophan, tyrosine, and the leucine group was established in one or more of the filtrates. In no case were more than four ninhydrin-positive substances detected in any individual filtrate. Indirect confirmation of the presence of specific amino acids could be deduced by comparison of the growth response of the 'A' group bacteria in the presence of a definite amino acid with the growth obtained upon the addition of cell-free filtrates found to contain that amino acid. A few examples of this relationship are given in Table IV.

In order to minimize the possibility that the amino acids found resulted from autolysis rather than from liberation from intact cells, the amino acid content of the cell-free culture filtrate in the basal medium was compared with the free amino acid content of the cells after their disruption by sonic

TABLE IV

GROWTH RESPONSE OF "A" GROUP BACTERIA CAPABLE OF GROWTH WITH A SINGLE AMINO ACID TO "B" GROUP FILTRATES KNOWN TO CONTAIN THAT AMINO ACID

| "A" group (Cult. No.) | Capable of growth with | | | "B" group filtrate known to contain | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Aspartic | | Proline | Leucine complex | | |
| | Aspartic | Proline | Leucine | F 56 | RCS 64 | T 28 | OR 52 | FR 79 | TR 4 |
| A 53 | – | X | X | – | – | X | X | – | – |
| OS 67 | – | – | X | X | X | X | X | X | X |
| RCS 30 | X | X | X | X | X | X | X | X | X |
| RCS 61 | X | – | X | X | X | X | X | X | X |
| T 39 | – | X | X | X | – | X | X | X | X |
| T 74 | – | – | X | X | X | X | X | X | X |
| TR 19 | – | X | – | X | – | X | – | – | – |
| W 21 | X | – | – | X | X | X | X | X | X |
| WR 10 | X | X | X | X | X | X | X | X | X |

NOTE: X = growth; – = no growth.

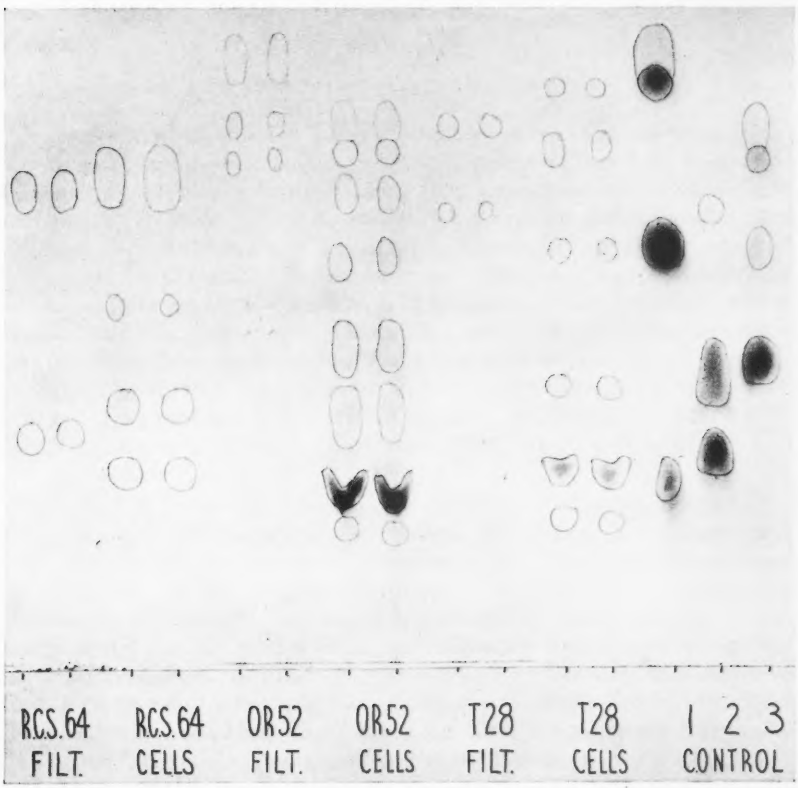


FIG. 1. Chromatogram of amino acid content of cell-free filtrates of 'B' group bacteria grown in Medium B for 3 days at 26° C. and the intracellular free amino acid content of the cells removed from the media.

Control 1. Histidine, alanine, leucine.
2. Aspartic, glutamic, proline.
3. Serine, tyrosine, phenylalanine.

vibration. For these tests the bacteria, which normally require 5 to 7 days for maximum growth, were harvested after 3 days. At this time the cultures were still in the logarithmic phase of growth and the possibility of an accumulation of autolytic products in the filtrate was greatly reduced. Fig. 1 is representative of the results obtained. The pattern obtained with the cell-free filtrates differs from that with the intracellular contents; the amino acids showing the greatest concentration within the cells may not even appear in the medium.

## Discussion

The preferential stimulation in the rhizosphere of bacteria requiring amino acids for maximum development may be attributed to an increased free amino acid content in the rhizosphere soil arising from a variety of sources. Lochhead and Thexton (7) considered the decomposition of organic, nitrogenous residues in the soil, the breakdown of sloughed off root material, and the excretion of amino acids by plants as possible sources. They also suggested that the preferential stimulation of the amino acid group might be concerned, at least partially, with the phenomenon of associative action between different groups of organisms, and reported that 46.7% of the cell-free filtrates of basal medium in which 'B' group bacteria had grown were capable of supporting the growth of 'A' group organisms. In the present studies it has been found that 40.6% of similar filtrates provided nutrients permitting the growth of amino acid requiring bacteria.

The isolation, from these filtrates, of a number of specific amino acids and other ninhydrin-positive substances supports the belief that the associative action is due to the occurrence of these substances. The results also suggest that the ability to liberate these substances is a fairly general property of soil bacteria with simple nutritional requirements. That the amino acid content of the medium results from their liberation from intact cells rather than from breakdown of dead cells is indicated by the limited number of amino acids found in any single filtrate, the ability of the filtrate to support only specific amino-acid-requiring bacteria, and the apparent differences between the intracellular free amino acid content of the bacteria and that of the medium in which they had grown.

The wide variations found in the nutritional requirements of the amino-acid-requiring bacteria in respect to their growth response to single amino acids and 'B' group filtrates indicate a nutritional complexity in this group, ranging from the ability to utilize a large number of individual amino acids to requirements for two or more specific acids. A possible inhibitory effect of certain combinations of amino acids or other antagonistic metabolic products is also indicated in cases in which an 'A' group organism known to utilize a specific amino acid failed to develop in a 'B' group filtrate known to contain that amino acid. The relatively high percentage of 'A' group organisms capable of utilizing methionine as a single amino acid source agrees with the results reported by Wallace and Lochhead (11).

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# THE METABOLISM OF YEAST SPORULATION

## II. STIMULATION AND INHIBITION BY MONOSACCHARIDES[1]

J. J. MILLER


## Abstract

Sporulation and growth of a bakers' yeast isolate were compared in 0.05 to 1% glucose, fructose, mannose, galactose, and dihydroxyacetone. Maximum yields of asci in glucose, fructose, and mannose were observed at 0.05% with a marked decline in increasing concentrations. Maximum yields of three- and four-spored asci were found at 0.1%. Galactose differed from the other hexoses in that the decline in ascus yields at higher concentrations was relatively small. The addition of 0.05% acetate increased the number of spores per ascus in the lowest concentrations of hexose. Cells grown in galactose were somewhat longer than glucose-grown cells, but not when $10^{-1}$ *M* phosphate, $3.3 \times 10^{-3}$ *M* arsenate, or 0.033% glucose were added to the growth medium. They also formed fewer spores per ascus than glucose-grown cells. Yields of asci in dihydroxyacetone were comparable to those observed in hexose, but growth in this compound was extremely slow. No sporulation or growth was observed in DL-glyceraldehyde, DL-glyceric acid, or glycerol.


## Introduction

Stantial (7) compared the sporulation of yeast in solutions of several sugars and found that asci formed in glucose, fructose, mannose, sucrose, and maltose when the concentration was 0.01 to 0.1%. With galactose, melezitose, and raffinose the upper limit for sporulation was much higher. Fructose, mannose, and galactose gave higher yields than glucose. No asci were found in arabinose, xylose, or lactose. Using slants of water agar with 0.05% of various sugars added, Miller *et al.* (2) observed that more asci formed with fructose and mannose than with glucose or galactose. The latter two sugars gave similar yields. In the preceding paper of this series (3) the declining yields of asci at glucose concentrations from 0.1 to 1.0% in phosphate buffer were investigated. It was inferred that the low yields were due to an inhibition since the addition of sodium acetate did not promote sporulation. The latter compound alone was very stimulating to sporulation at all concentrations from 0.01 to 1.0%, although it supported growth much less effectively than did glucose. It was further noted that the asci formed in glucose solutions were mostly two-spored, whereas in acetate three- and four-spored asci tended to predominate. An approach was made toward determining the mechanism of sporulation inhibition at the higher concentrations of glucose by comparing sporulation and growth in glucose, pyruvate, acetaldehyde, ethanol, and acetate, and by using various inhibitors known to have more or less definite metabolic effects. The results indicated that the inhibition was due to glucose itself or to some metabolic product arising from glucose above the level of pyruvate. The present contribution describes

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further experiments on the stimulation and inhibition of sporulation by monosaccharides, done with a view to assessing the relation of the preliminary stages of carbohydrate metabolism to the process.

## Materials and Methods

Cells for preparing sporulation and growth experiments were harvested from 2-day cultures in 6-oz. pharmaceutical bottles that contained 10-ml. volumes of a presporulation medium consisting of yeast nitrogen base (Difco) prepared in *M*/30 phthalate buffer of pH 5 with 1% glucose added. Sporulation and growth media were also prepared with *M*/30 phthalate buffer of pH 5. Both sporulation and growth cultures were 8 ml. in volume. A typical growth culture contained: 2 ml. of 0.2% glucose + 2 ml. yeast nitrogen base of four times the concentration recommended by the manufacturer + 2 ml. buffer + 2 ml. of a cell suspension in buffer with approximately 400,000 cells per milliliter; and a typical sporulation culture: 2 ml. 0.2% glucose + 4 ml. buffer + 2 ml. of a cell suspension with approximately 16,000,000 cells per milliliter. When other substances such as arsenate or phosphate were to be included, they were added as 2 ml. of a solution four times the desired final strength in buffer. The cell suspension was always the last component added. Growth was determined 1 day, and sporulation 3 to 5 days after the cultures were prepared. Controls with the carbon source omitted were included in all experiments. Sporulation was always slight under these conditions, rarely exceeding 1%. The cell population density in the controls in growth experiments usually increased two to four times, and these values were deducted in preparing the graph of the growth experiments (Fig. 9). Other procedures were as described by Miller and Halpern (3).

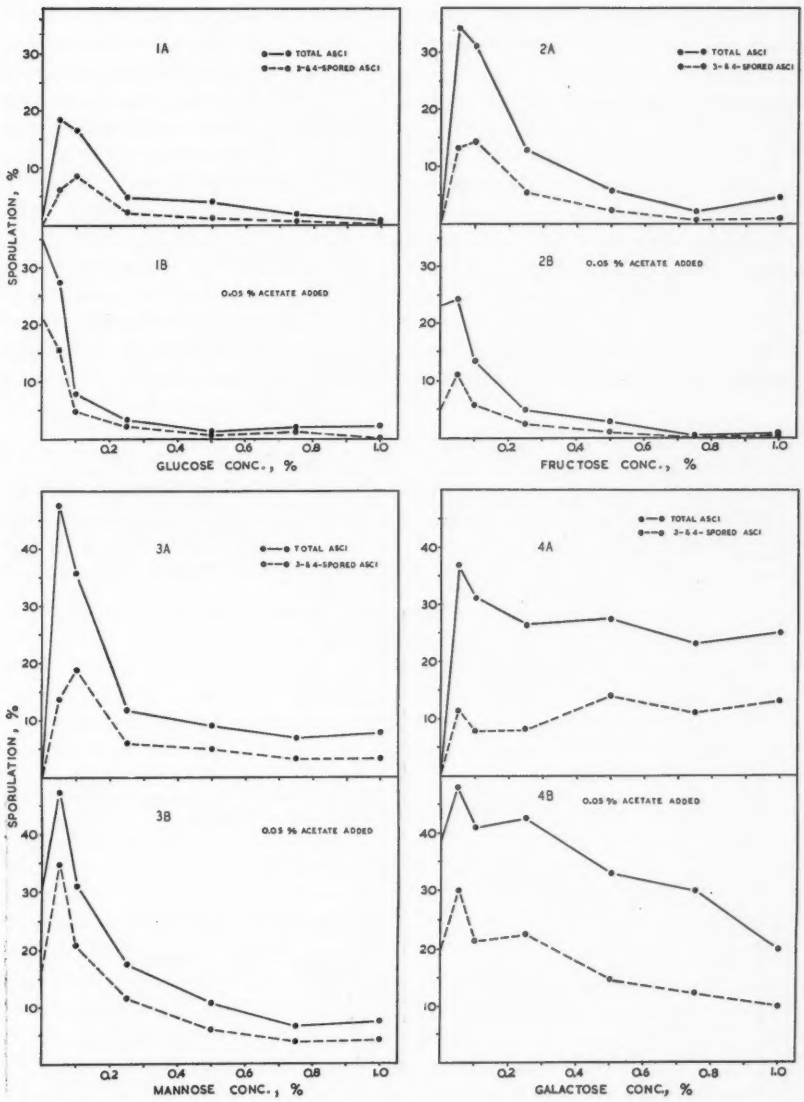
## Results

*Sporulation in Glucose, Fructose, Mannose, and Galactose*

Sporulation was compared in six concentrations of each hexose (0.05, 0.1, 0.25, 0.5, 0.75, 1.0%), and the results are shown in Figs. 1A, 2A, 3A, 4A. With glucose, fructose, and mannose the highest yields of asci were found at 0.05%, with a decline at the greater concentrations. On the average, yields were greater in fructose, mannose, and galactose than in glucose. Sporulation in galactose differed in that yields showed a comparatively small decline with increasing hexose concentration.

With all four sugars the proportion of three- and four-spored asci was similar, and comprised about half of the total except at the lowest concentrations. The proportion of such asci showed a distinct increase as the concentrations of glucose, fructose, and mannose were raised from 0.05 to 0.1%. With galactose an increase seems apparent between 0.1 and 0.5%.

Figs. 1B, 2B, 3B, and 4B show the results of repeating these experiments with the inclusion of 0.05% sodium acetate in all cultures. Since the two series of experiments were not done simultaneously, the results are not strictly




FIGS. 1–4. Yields of asci and of three- and four-spored asci in varying concentrations of four monosaccharides, with and without the addition of 0.05% sodium acetate.

comparable. There is, however, the indication that addition of 0.05% acetate to varying concentrations of glucose, fructose, and mannose did not increase yields except, possibly, at the lowest glucose concentration (0.05%). With galactose, the acetate seemed to increase yields at all concentrations except the highest. As usual, acetate alone was very effective as a nutrient substrate for sporulation, and good yields of asci were found in the controls containing 0.05% acetate. These yields were increased by the addition of 0.05% mannose or galactose and decreased by 0.05% glucose. It is of interest that the low proportion of three- and four-spored asci noted above at the weaker hexose concentrations was not apparent when 0.05% acetate was included. The acetate also seemed to increase somewhat the proportion of three- and four-spored asci at higher hexose concentrations, but this was not consistent.

In a further experiment 0.3% acetate was combined with varying concentrations of glucose, and the results were similar to those shown in Fig. 1B. That is, additional acetate did not overcome the decline in sporulation caused by increasing the glucose concentration.

*Sporulation in Dihydroxyacetone*

Two trioses, dihydroxyacetone and DL-glyceraldehyde, were also studied. The latter did not stimulate sporulation at any concentration tested and the inclusion of 0.1 or 4.5% of monobasic potassium phosphate in the cultures with the glyceraldehyde had no effect. In contrast, dihydroxyacetone compared quite favorably with the hexoses as a sporulation stimulant (Fig. 5). It

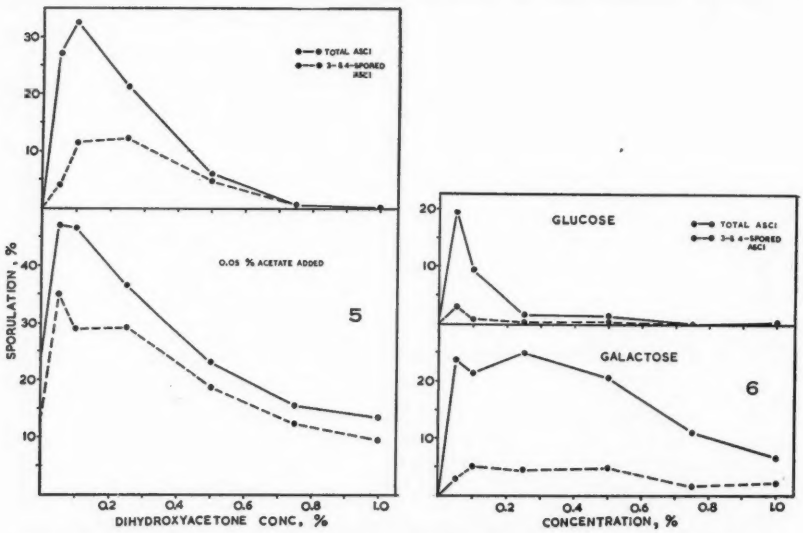



FIG. 5. Yields of asci and of three- and four-spored asci in varying concentrations of dihydroxyacetone, with and without the addition of 0.05% sodium acetate.

FIG. 6. Yields of asci and of three- and four-spored asci obtained with galactose-grown cells in varying concentrations of glucose and galactose.

differed from them, however, in having the optimum at 0.1% and in that the addition of 0.05% acetate increased sporulation at all concentrations. The proportion of three- and four-spored asci increased as the dihydroxyacetone concentration was raised, but in the presence of 0.05% acetate this proportion was high at all concentrations.

No stimulation of sporulation was obtained with solutions of DL-glyceric acid or glycerol.

*Sporulation of Galactose-grown Cells*

For these experiments cells were multiplied in presporulation medium containing 1% galactose instead of 1% glucose. They were then placed in sporulation solutions containing glucose or galactose. From the results, shown in Fig. 6, it seems evident that the yields of asci in glucose were similar to those obtained above (Fig. 1) with glucose-grown cells. In galactose, yields were somewhat less than with glucose-grown cells (Fig. 4). The proportion of three- and four-spored asci formed in glucose or galactose was less than with glucose-grown cells.

It has been shown (5) that considerable quantities of the galactose consumed by yeast may be stored as reserves such as glycogen instead of being completely metabolized. Attempts were made to alter the proportion of three- and four-spored asci by treatments that would be expected to diminish the amounts of such reserves in the cells, i.e., by starvation and by treatment with arsenate and phosphate.

In the starvation experiment galactose-grown cells were used to prepare sporulation cultures which were incubated for periods of 0 (control), 1, 2, 5, 7, and 9 days before the nutrient substrate, 0.05% glucose, was added. This treatment did not affect the relative abundance of three- and four-spored asci. It is of interest that 5 days of starvation did not diminish the ability of the cells to sporulate. But the yield from cells starved for 7 days was 44% of the control.

The results of an experiment in which arsenate and phosphate were added to the presporulation and sporulation media are summarized in Table I. Phosphate greatly increased the proportion of three- and four-spored asci in galactose-grown cells sporulating in galactose, but arsenate did not. When arsenate was included in the presporulation medium, the proportion of three- and four-spored asci formed in galactose was increased 10-fold, and the further inclusion of arsenate in the sporulation medium gave no additional increase, whereas phosphate apparently did so. Cells from presporulation medium containing phosphate gave only 10.5% sporulation, and the further addition of arsenate or phosphate to the sporulation medium decreased the yield more. These cells did not produce more spores per ascus than those from presporulation medium lacking phosphate, but there is an indication that they formed three- and four-spored asci more frequently when phosphate or arsenate was added to the sporulation medium. These results indicate that arsenate is more effective than phosphate in increasing the number of spores

TABLE I

EFFECT OF ARSENATE AND PHOSPHATE ON SPORULATION OF GALACTOSE-GROWN CELLS IN 0.05% GALACTOSE

| Presporulation medium | Sporulation medium | Sporulation | |
|---|---|---|---|
| | | Total asci, % | 3- and 4-spored asci, % |
| Yeast nitrogen base | Buffer only | 0.3* | |
| + 1% galactose | 0.05% galactose | 27.0 | 1.3† |
| | 0.05% galactose + $3.3 \times 10^{-3}$ *M* $Na_2HAsO_4$ | 14.5 | 1.8 |
| | 0.05% galactose + $3.3 \times 10^{-1}$ *M* $KH_2PO_4$ | 19 | 6.8 |
| Yeast nitrogen base | Buffer only | 0.3 | |
| + 1% galactose | 0.05% galactose | 25.5 | 14.0 |
| + $3.3 \times 10^{-3}$ *M* $Na_2HAsO_4$ | 0.05% galactose + $3.3 \times 10^{-3}$ *M* $Na_2HAsO_4$ | 18.3 | 10.0 |
| | 0.05% galactose + $3.3 \times 10^{-1}$ *M* $KH_2PO_4$ | 22.0 | 17.2 |
| Yeast nitrogen base | Buffer only | 0.3 | |
| + 1% galactose | 0.05% galactose | 10.5 | 0.95 |
| + $3.3 \times 10^{-1}$ *M* $KH_2PO_4$ | 0.05% galactose + $3.3 \times 10^{-3}$ *M* $Na_2HAsO_4$ | 5.3 | 1.1 |
| | 0.05% galactose + $3.3 \times 10^{-1}$ *M* $KH_2PO_4$ | 6.3 | 1.9 |

*Each figure based on an examination of 400 cells.
†Each figure based on an examination of 200 asci.

per ascus (formed by galactose-grown cells sporulating in galactose) when added to the presporulation medium, and that the reverse is true when they are added to the sporulation medium.

Galactose-grown cells have proved capable of forming three- and four-spored asci fairly abundantly in acetate. In three experiments they formed an average of 17.9% three- and four-spored asci in 0.05% acetate, with a total sporulation of 36.4%, and this compares quite well with the usual results obtained with glucose-grown cells.

*Sporulation in Pyruvate and Acetate*

For comparison with the sporulation in the monosaccharides, results obtained with the same range of concentrations of pyruvate and acetate are shown in Figs. 7 and 8. The former substance gave the highest yields of asci obtained during this study, and there was no definite decrease at the higher concentrations. The optimum concentration for sporulation with acetate was 0.25%. As with the monosaccharides, the number of spores per ascus tended to be greater as the concentration was increased, but here the proportion of three- and four-spored asci was, on the average, much higher.

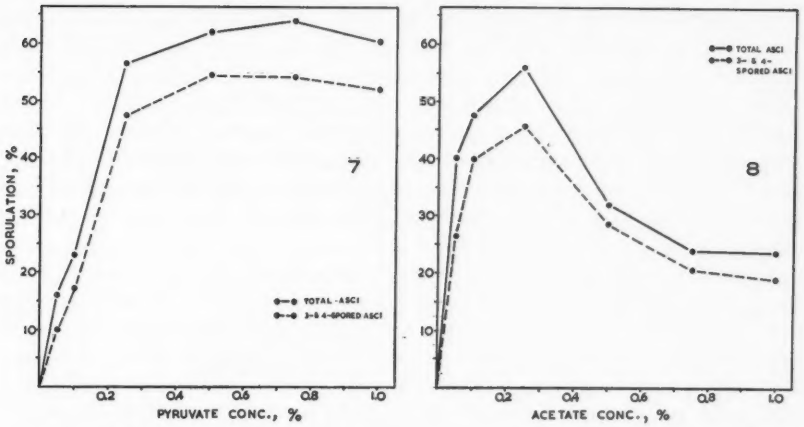



FIG. 7. Yields of asci and of three- and four-spored asci in varying concentrations of sodium pyruvate.
FIG. 8. Yields of asci and of three- and four-spored asci in varying concentrations of sodium acetate.

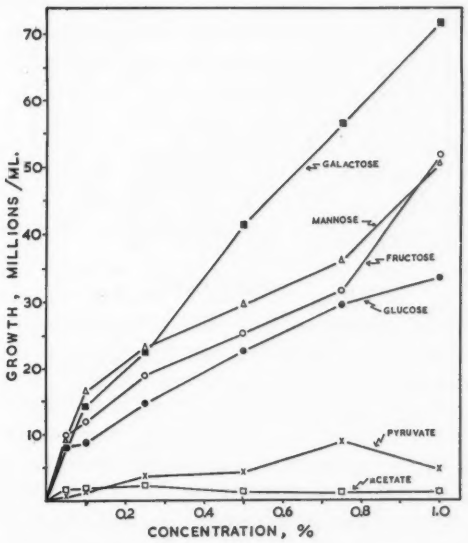



FIG. 9. Increase in cell population density in 1 day in growth medium containing four hexoses, pyruvate, and acetate in varying concentrations.

*Growth in Monosaccharides, Pyruvate, and Acetate*

Growth experiments were done in conjunction with most of the above sporulation experiments, and the results are compared in Fig. 9. The four hexoses gave far more growth in 1 day than acetate or pyruvate. More growth was observed in acetate than in pyruvate at the two lowest concentra-

tions, and the reverse was found at the higher concentrations. At concentrations of 0.5 to 1.0%, galactose was the best substrate for growth, which indicates that its utilization is not adaptive in this strain of yeast. Supporting evidence was obtained in a growth experiment with the four hexoses in 1% concentration during which the rate of cell multiplication in the first few hours was compared. Although the cells had not previously grown in galactose, growth was not less rapid with this sugar than with the others, and after 24 hours cells were nearly twice as abundant in galactose as in any of the other sugars.

Repeated experiments have failed to give definite evidence of growth in 1 day with dihydroxyacetone. After 3 days, however, cell numbers have been observed to increase from 100,000 to 1–2 million per milliliter with the higher concentrations used. Such slow growth is noteworthy in view of the fact that dihydroxyacetone is quite effective as a nutrient substrate for sporulation. The addition of monobasic potassium phosphate ($3.3 \times 10^{-1}$ *M*) to the growth medium did not accelerate growth in dihydroxyacetone. DL-Glyceraldehyde, DL-glyceric acid, and glycerol were not found to support growth but, as mentioned above, these substances did not support sporulation either.

Cells grown with galactose as the nutrient substrate were more elongate than those grown in glucose or in any of the other compounds used here. Moreover, the buds tended not to separate readily from the mother cells so that small groups of cells were frequent. Average dimensions of cells grown for 1 day in 0.05% galactose were: $7.4\ \mu \times 4.1\ \mu$; and in 0.05% glucose: $6.5\ \mu \times 4.6\ \mu$. When $3.3 \times 10^{-3}$ *M* arsenate or $10^{-1}$ *M* phosphate was added to growth medium containing 0.05% galactose, the cells produced had the usual oval to round shape. Likewise, the addition of 0.033% glucose to growth medium containing 0.1% galactose restored the round shape.

## Discussion

In a study of the respiration of bakers' yeast at different concentrations of glucose, Aldous *et al.* (1) found that below 0.09% the rate of carbon dioxide production was the same as the rate of oxygen uptake, but above this level it exceeded the oxygen uptake. They interpreted this to mean that the oxygen-consuming system does not operate rapidly enough to remove all the fermentation products produced in higher glucose concentrations. Their results afford an interesting parallel to the decrease in ascus yields observed here in concentrations of glucose above 0.05%, and support the suggestion of Miller and Halpern (3) that the decline is due to an accumulation of some metabolic intermediate or intermediates formed from glucose. Aldous *et al.* concluded that ethanol was the main fermentation product that accumulated in their cultures, but it seems improbable that this substance is the cause of the low ascus yields since pyruvate (1%), which should form ethanol as readily as glucose, did not depress sporulation.

The failure to observe strong inhibition of sporulation with galactose may be related to the fact that whereas glucose, fructose, and mannose are

phosphorylated in yeast by hexokinase, a different enzyme, galactokinase, phosphorylates galactose (8). Possibly galactose, in consequence, does not enter the Embden–Meyerhof pathway rapidly enough to cause inhibiting amounts of intermediate compounds to accumulate. This hypothesis receives some support from the observation that mannose, which was not as inhibiting to sporulation as glucose, has been found by Slein *et al.* (6) to be phosphorylated less rapidly by hexokinase than glucose at concentrations that saturate the enzyme. An alternative explanation is that the inhibition arises through the entrance of some of the glucose into another metabolic pathway which is not concerned in sporulation, and which is not so readily available to galactose. Rothstein (4) demonstrated that there are fewer kinds of sites on the surface of the yeast cell capable of taking up galactose than glucose.

Cells grown in galactose, in addition to being more elongate than those grown in glucose, were less capable of forming three- and four-spored asci. Since phosphate and arsenate counteract both effects, they may be linked in some manner. The observation that a small amount of glucose restored the round shape of cells growing in galactose indicates that galactose-grown cells may lack some metabolite more readily formed from glucose. Explanation of the differences observed in sporulation and growth with glucose and galactose must await further study.

Three- and four-spored asci always became more abundant when the concentrations of the various nutrients were increased, but the proportion of such asci was never so high in the hexoses as in acetate or pyruvate. Moreover, the addition of 0.05% acetate to the hexose solutions or to dihydroxyacetone increased the number of spores per ascus at the lowest concentrations. Possibly the oxygen-consuming system is important in determining the number of spores per ascus, and in dilute hexose the formation of substances such as pyruvate or acetate that can serve more or less directly as substrates for respiration may not be sufficiently rapid to allow the maximum rate of respiration. This is unlikely to be the complete explanation, however, since at the higher hexose concentrations, acetate seemed less effective in increasing the number of spores per ascus. Miller and Halpern (3) made similar observations and suggested that an inhibition of three- and four-spored ascus formation by glucose was involved.

Dihydroxyacetone was unique among the sporulation substrates studied here and in the preceding paper in failing to support active growth. This is not due to a growth inhibition since it has been found to allow the normal rate of cell multiplication when included in growth medium with glucose, galactose, pyruvate, or acetate. A possible explanation for the anomalous results with dihydroxyacetone is that at least one of the pathways of carbohydrate metabolism in the yeast cell is capable of promoting sporulation but not growth, and that this is the only pathway by which dihydroxyacetone can enter. Miller and Halpern (3) referred to the inhibition of sporulation in acetate by low concentrations of ammonium sulphate, and suggested that the inhibition might result from a competition between synthetic reactions

concerned in sporulation and growth for metabolites formed from the acetate, which is determined in favor of growth by ammonium. During the present study $10^{-3}$ *M* ammonium sulphate has been found to give almost complete inhibition of sporulation in glucose, galactose, pyruvate, acetate, and ethanol but it did not depress sporulation in dihydroxyacetone. This is not at variance with the hypothesis that dihydroxyacetone is confined to a metabolic pathway that is not connected with growth.

## Acknowledgments

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# YELLOW-PIGMENTED PATHOGENIC MYCOBACTERIA FROM CERVICAL LYMPHADENITIS[1]

F. H. Prissick and A. M. Masson

## Abstract

Among 14 strains of yellow-pigmented acid- and alcohol-fast rods isolated from human pathological materials, 10 strains from tuberculoid lesions of cervical or facial lymphadenitis in children merit particular interest. They were compared with some of the mycobacteria, including *Mycobacterium tuberculosis*, and with *Nocardia*. On the basis of morphology, staining properties, cultural characteristics, pathogenicity, and allergic reactions in laboratory animals, they have been placed in the genus *Mycobacterium*. However, the degree and quality of their differences from other named species of the genus as observed under the present experimental conditions, and the fact of their isolation in pure culture from closed lesions, has led to the proposal that they be given the name *Mycobacterium scrofulaceum* n. sp.

Chromogenic strains of acid-fast rods have been isolated from a wide variety of sources including human pathological materials such as sputum, lung tissue, gastric lavage, and bronchial aspiration fluid. Some have proved to be contaminants belonging to the saprophytic group of mycobacteria (6, 7, 11, 12, 13). The role of other strains in the pathogenesis of the disease with which they were associated has been difficult to assess, particularly since the majority of them were obtained from open lesions (1, 2, 4, 7, 8, 14, 16) although occasionally acid-fast rods which are not *Mycobacterium tuberculosis* have been obtained from closed lesions, notably *Mycobacterium fortuitum* (3, 15). In 1951 (9), a preliminary report was made on 14 strains of chromogenic acid and alcohol-fast rods, 10 of which were isolated in pure culture, from pus aspirated from suppurating lymph nodes of the cervical region in children suspected of tuberculous infection. The 10 strains obtained from closed lesions were considered to be of pathological significance. The four remaining strains were cultured from two empyema fluids, a bronchial aspiration and tissue from a tuberculous infection of the knee joint, and have not been included in the present study.

### *Sources of the Strains*

In all instances the specimens consisted of pus aspirated under aseptic conditions from non-ulcerated facial, submaxillary, or cervical lymph nodes in young children:* No. 526 (I), 1356 (II), 1637 (III), 2465 (IV), and 3327 (V) were isolated at the Montreal Children's Hospital. No. 623 (VI) was isolated at the Royal Victoria Hospital, Montreal. No. 3536 (VII), 4333 (VIII), 5099 (IX), and 8053 (X) were isolated at the Hospital for Sick Children, Toronto. These strains will be referred to subsequently as strains I to X.

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Contribution from the Montreal Children's Hospital, and the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec.

*A more detailed account of the case histories and a discussion of the role of the chromogenic mycobacteria in the pathogenesis of disease will appear separately (10).

Short acid- and alcohol-fast rods were seen in smears of the aspirated pus in 6 out of the 10 cases.

*Primary Isolation*

Bright yellow colonies appeared between the 4th and 5th week of incubation on Loewenstein's slopes at 37° C., and the color deepened with age. All strains were smooth. The colonies were 1–2 mm. in diameter, convex, opaque, glistening, with entire edge; the growth on stroke subcultures was slightly confluent and papillate on further incubation. They emulsified easily in physiological saline to give homogenous suspensions.

## Experimental Studies

### Morphology and Staining Reactions

Smears were made from 10-day-old cultures incubated at 37° C. on the following media: Loewenstein's, glycerin egg, glycerin broth, litmus milk, and Dubos' medium. Compared with *M. tuberculosis*, the rods examined from growth on the solid media were often thicker in diameter, shorter, and frequently beaded. In Dubos' medium, some strains showed cord formation; many of the rods were filamentous. In litmus milk, variation in form was present in all strains, and particularly marked in some (II, III, V). There were differences in shape and size: filamentous forms were numerous; many showed beading, clubbing of the ends, citron shaped swellings, and irregularity of staining, but no branching was seen in any strain.

The rods stained Gram-positive, although unevenly, giving a beaded appearance. Stained by the Ziehl-Neelsen method, all strains were strongly acid- and alcohol-fast and resisted decolorization with 25% sulphuric acid and 95% ethyl alcohol for 10 minutes each. When the cultures were kept for 3 weeks, five strains (I, II, III, V, VI) resisted this decolorizing process for 60 minutes, the remainder varied in their retention of stain.

### Cultural Studies

The following media were inoculated with every strain: Peptone agar, 4% human blood agar, 5% sheep blood agar, 5% rabbit blood agar, Bordet-Gengou, Sabouraud's dextrose agar, Loewenstein's, Dorset's egg, 5% glycerin peptone agar, 5% glycerin tryptose agar, Loeffler's serum, potato, 5% glycerin peptone broth, 5% glycerin peptone broth with 0.5% phenol, Dubos' medium, beef infusion broth, litmus milk, and Czapek's medium with paraffin-coated rod. The paraffin used was "Tissue Mat" (Fisher Scientific). The strains were tested for their ability to utilize glucose, saccharose, lactose, maltose, mannitol, trehalose, arabinose, sorbitol, inulin, and rhamnose, using 1% of each sugar in Hiss serum water, with Andrade's indicator. Strains were also tested for indol production, reduction of nitrates, production of hydrogen sulphide, liquefaction of gelatin, and hydrolysis of starch. *Mycobacterium tuberculosis* (H37Rv) was inoculated into the same media for comparison.

The inoculum in each case consisted of one drop (approximately 0.05 ml.) of a suspension of the bacteria in 0.85% saline standardized so as to contain $13 \times 10^8$ organisms per ml. The stock suspension was prepared from two-week-old cultures on Loewenstein's medium. Growth was observed weekly for a period of 6 weeks' incubation at 37° C.

*Results*

The strains grew on the majority of these media. Growth of all strains was most profuse on Loewenstein's, Dorset's egg, Dubos', Bordet-Gengou, and 5% glycerin agar. On Loewenstein's medium, growth was observable within 2 weeks, the distinct yellow pigment becoming pronounced after 3 weeks at 37° C. Dubos' medium was slightly turbid within a week, becoming more opaque with a slimy homogenous yellowish sediment at the end of the second week. No strain grew in glycerin broth containing 0.5% phenol. On peptone agar or broth, strains grew thinly or not at all. H37Rv showed a scanty growth on peptone agar, and none in beef infusion broth. Seven strains failed to grow on Loeffler's serum slopes, strain No. IV grew in 2 weeks, I and II in 4 to 6 weeks, and H37Rv grew poorly in 2 weeks. Eight strains grew in Czapek's medium, covering the paraffin-coated rod in 3 to 4 weeks; the remainder, III and VI, failed to grow in this medium, as did H37Rv. Growth was present in Hiss serum water sugars, but no acid was produced from any of them. Gelatin was not liquefied, indol was not formed, and $H_2S$ was not produced.

Growth at different temperatures was observed on Loewenstein's and Dubos' medium during a 6 week period. All strains grew best at 37° C. No strain grew at 47° C. or at 50° C. They all grew at room temperature in 2 to 3 weeks.

All strains, including H37Rv and excepting X, grew readily in an atmosphere containing 15% carbon dioxide and did not grow under anaerobic conditions.

Seven strains survived exposure of 1 hour at 56° C., four survived exposure to 60° C. for 30 minutes, and all were killed at 100° C. for 1 minute. H37Rv was killed under all three conditions.

Three strains failed to grow following 20 hours' exposure to 17% trisodium phosphate; all grew following one-half hour exposure to 4% NaOH and 3 hours' exposure to 3% HCl.

*Antibiotic Sensitivity Tests*

Sensitivity to para-aminosalicylic acid incorporated in Herold's egg yolk medium: five strains were inhibited by 10 mcg., three by 100 mcg., and two by 1000 mcg.

Sensitivity to streptomycin incorporated in Dubos' fluid medium: two strains were not inhibited by 2.0 mcg., three were inhibited by 2.0 mcg., two by 1 mcg., and three by 0.7 mcg. H37Rv was sensitive to 0.5 mcg. of streptomycin and 1 mcg. of para-aminosalicylic acid.

*Neutral Red Reaction*

The method used in the present study is described by Morse *et al.* (5). All strains gave negative results.

Some of the characteristics of the 10 strains of mycobacteria are presented in Table I. Table II depicts some features of our strains in comparison with other members of the order Actinomycetales.

TABLE I

| Source, cervical gland | | Colony type | Antibiotic sensitivities | | Resistance to heat | | Resistance to digestants | | | Neutral red test |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Strepto-mycin | P.A.S. | 1 hour, 56° C. | 30 min., 60° C. | 4% NaOH 30 min. | 3% HCl 3 hours | 17% $Na_3PO_4$ 20 hours | |
| I | 526 | Smooth | 1 mcg. | 10 mcg. | + | + | + | + | + | 0 |
| II | 1356 | Smooth | >2 mcg. | 100 mcg. | 0 | 0 | + | + | + | 0 |
| III | 1637 | Smooth | 0.7 mcg. | 10 mcg. | 0 | 0 | + | + | 0 | 0 |
| IV | 2465 | Smooth | 0.7 mcg. | 10 mcg. | + | + | + | + | + | 0 |
| V | 3327 | Smooth | 2 mcg. | 1000 mcg. | 0 | 0 | + | + | 0 | 0 |
| VI | 623 | Smooth | >2 mcg. | 10 mcg. | + | 0 | + | + | + | 0 |
| VII | 3536 | Smooth | 2 mcg. | 10 mcg. | + | 0 | + | + | + | 0 |
| VIII | 4333 | Smooth | 2 mcg. | 100 mcg. | + | 0 | + | + | + | 0 |
| IX | 5099 | Smooth | 1 mcg. | 100 mcg. | + | + | + | + | + | 0 |
| X | 8053 | Smooth | 0.7 mcg. | 1000 mcg. | + | + | + | + | 0 | 0 |
| Control H37Rv | | Rough | 0.5 mcg. | 1 mcg. | 0 | 0 | + | + | + | + |

TABLE II

| Strains | Pigmentation | Acid-alcohol fastness | Branching | Phenol tolerance | Growth on paraffin | Pathogenicity G.P. |
|---|---|---|---|---|---|---|
| *Nocardia asteroides* | + | ± | + | − | + | + |
| *Nocardia intra-cellularis* | + | + | + | + | + | + |
| *M. phlei* | + | + | − | − | + | − |
| *M. scrofulaceum* n. sp. | + | + | − | − | + | + |
| H37Rv | − | + | − | − | − | + |

## Animal Experiments

The 10 strains were inoculated into rats, hamsters, guinea pigs, and hens. The inoculum consisted of a 2-week-old culture on Loewenstein's medium suspended in 0.85% saline and standardized to contain approximately $13 \times 10^8$ organisms per ml. For each strain, subcutaneous inoculations of 0.25 ml. of the suspension were made into two rats and one or two hamsters; two guinea pigs received 0.5 ml.; intraperitoneal inoculations using the same dosages were made into two rats, two hamsters, and two guinea pigs; intravenous inoculations of 0.5 ml. were made into two hens.

Skin tests were done prior to inoculation and 2 months afterwards. Rats, hamsters, and guinea pigs were tested on the abdomen with human O.T. diluted 1/100, avian tuberculin diluted 1/500, and homologous "chromogenic extract" in a 1/10 dilution. Hens were tested on the wattles with avian tuberculin and homologous "chromogenic extract" only.

The "chromogenic extracts" were prepared by growing each strain in Sauton's medium for 5 weeks at 37° C.; the organisms were then killed by boiling and the culture was evaporated to 1/10th of its original volume. After centrifuging, the clear supernatant was bottled aseptically and diluted 1/10 with 0.85% saline before use.

Only animals which gave negative reactions to all materials on primary testing were chosen for the experiments. The animals were kept under observation for 3 months; although a few animals in each group died during the 3 months' observation period, there were no essential differences in the postmortem findings for these animals as compared with those that were killed at the end of the 3 months. All animals were autopsied, smears were made from any lesions found and stained by the Ziehl-Neelsen method, and cultures were made to recover the strains from any lesion which afforded sufficient material. Organs were kept for sections whenever gross lesions were observed; their histological examination was not complete at the time of writing.

*Results*

*Rats Inoculated Subcutaneously*

No abscess or ulceration at the site of inoculation was found in any animal; several of them had small, calcified nodules in which acid–alcohol fast rods were found on smear, but which were not considered as true lesions. Purulent lymph nodes were found in the omentum, and acid–alcohol fast rods were present on smears in animals inoculated with strains II (one animal), III (one animal), VIII (one animal), and IX (one animal). No lesions were found in the spleen, liver, kidneys, or lungs.

*Rats Inoculated Intraperitoneally*

Thickening of the omentum and enlarged lymph nodes were observed in animals inoculated with strains II (one animal), III (one animal), V (one animal), and VIII (one animal). Acid–alcohol fast rods were demonstrated in smears of the lymph nodes. A lung abscess, with acid–alcohol fast rods in the smear, was found in one animal inoculated with strain II.

*Skin tests.*—Skin tests on these rats were all negative 2 months after inoculation with human O.T. and avian O.T.; slightly positive reactions were found with four extracts: I, II, III, VIII; doubtful reactions with extracts of IV, V, and X; negative reactions with extracts VI, VII, and IX.

*Hamsters Inoculated Subcutaneously*

No lesions were observed at the site of inoculation or enlargement of the local lymph nodes, but lesions in the abdominal cavity were observed in animals inoculated with strains IV (one animal had a purulent lymph node in the omentum), VIII (one animal had an enlarged lymph node in the omentum and an enlarged liver), V and X (one animal of each of these strains had a liver studded with nodules). Acid–alcohol fast rods were found in all lesions.

*Hamsters Inoculated Intraperitoneally*

Small nodules were found in the omentum, and the spleen was enlarged but without granulations. Acid–alcohol fast rods were demonstrated on smears from the nodules and the spleen in animals inoculated with the following strains: II (one animal), III (one animal), IV (one animal), V (one animal,) and VIII (one animal). The liver was enlarged and presented granulations in animals inoculated with strains IV (one animal) and VIII (one animal). A haemorrhagic pleural effusion was observed in one animal but no acid-alcohol fast rods were found.

*Skin tests.*—No reactions were obtained in these hamsters with human O.T. or avian O.T. Doubtful reactions were noted with chromogenic extracts of five strains (I, II, IV, VIII, X) and no reactions were seen with the extracts of the five other strains.

*Guinea Pigs Inoculated Subcutaneously*

Abscesses at the site of inoculation were observed in both animals inoculated with strains V, VII, and IX. Necrosis at the site of inoculation was noted in one animal inoculated with strain III. Regional lymph node involvement was found in one animal inoculated with strain IV and one animal inoculated with strain VIII. Thickening of the omentum was present following inoculation of strains IV (one animal), III (two animals), VI (two animals), VIII (one animal). Acid–alcohol fast rods were demonstrated on smears in all cases.

*Guinea Pigs Inoculated Intraperitoneally*

Regional lymph node enlargement with suppuration was the most constant and prominent of all the pathological findings. Various types and degrees of peritonitis were associated: thickening of the omentum, adhesions, and purulent or haemorrhagic effusion. Acid–alcohol fast rods were easily demonstrated on smears made from lymph nodes. Both animals showed such lesions in most instances. However, both animals injected with strain II died 2 months after inoculation, and in only one of them a small infected lymph node was found in the omentum; one animal died a few days after inoculation with strain VI, the other died 1 month later, and the only lesion found was in a small lymph node in the omentum, which was kept for histological section. Enlargement of the liver and granulations were infrequent; discrete granulations of the spleen were observed only with strains IV (two animals) and VIII (two animals). A haemorrhagic pleural effusion was found in one animal of each pair inoculated with strains IV, V, and IX, but no acid–alcohol fast rods were demonstrated on smears, or grown on culture.

*Skin tests.*—A doubtful reaction to avian O.T. was found in two guinea pigs inoculated with strain IV; a doubtful reaction to human O.T. was found in one animal injected with strain III and one animal injected with strain VI. Positive reactions to "chromogenic extracts" were elicited in almost all animals tested and with all the strains; however, no positive result has been recorded with strain II, the four animals having died before skin tests were made.

*Hens Were Given Intravenous Injections of the Various Strains.*

One of the two animals inoculated with strain VII died 3 months after inoculation; at autopsy, pleural and pericardial effusions and caseous areas in both lungs were found, and acid-alcohol fast rods were seen on direct smear. The second animal showed a strong reaction to avian tuberculin and "chromogenic extract" but no lesions were demonstrated at autopsy. All the other animals were killed 3 months after inoculation but no gross lesions were found in any of them.

*Skin tests.*—Skin tests were performed on the hens 3 months after inoculation. Positive reactions to avian O.T. were found in both animals inoculated with strains I, VI, VIII and in one animal only with strains III, IV, VII, IX, X; the reaction was doubtful in one animal inoculated with strains III and V; it was negative in both animals inoculated with strain II. Positive reactions to chromogenic extracts were obtained in both animals inoculated with strains I, III, VI, VIII, X; in one animal only with strains II, IV, VII, IX; and a doubtful reaction in one animal inoculated with strain V.

*In summary,* the following information has been obtained from the animal experiments. Hens showed allergic reactions with both avian tuberculin and chromogenic extracts but failed to develop lesions except in one isolated case. Hamsters failed to show definite allergic reactions to the "chromogenic extracts" but several developed both localized and generalized lesions. Rats showed definite allergic reactions to several of the "chromogenic extracts" and some developed localized lesions. Guinea pigs were the animals that developed lesions and showed allergic reactions to the chromogenic extracts most consistently. The lesions were more pronounced in animals inoculated into the peritoneal cavity than in those inoculated subcutaneously. They differed from tuberculous lesions by showing little tendency to be granulomatous in the spleen, liver, kidneys, or lungs. The infection seemed to progress by the lymphatic route only. It can be stated that an inoculum of $6.5 \times 10^8$ organisms of every strain studied has produced lesions in the guinea pigs inoculated by the intraperitoneal route.

A condensed summary of the animal experiments is presented in Table III.

TABLE III

SUMMARY OF PATHOLOGICAL FINDINGS AND ALLERGIC REACTIONS

| | Rats | | Hamsters | | Guinea pigs | | Hens | |
|---|---|---|---|---|---|---|---|---|
| Strains | Lesions | + skin test | Lesions | ± skin test | Lesions | + skin test | Lesions | + skin test |
| I (526) | 0/4 | 3/4 | 0/4 | 1/1 | 3/4 | 1/2 | 0/2 | 2/2 |
| II (1356) | 3/4 | 4/4 | 1/3 | 1/2 | 1/4 | Not done | 0/2 | 1/2 |
| III (1637) | 2/4 | 3/4 | 1/3 | 0/2 | 4/4 | 1/1 | 0/2 | 2/2 |
| IV (2465) | 0/4 | 3/4 | 3/3 | 1/1 | 4/4 | 2/2 | 0/2 | 1/2 |
| V (3327) | 1/4 | 2/4 | 2/3 | 0/2 | 3/4 | 1/1 | 0/2 | ±1/2 |
| VI (623) | 0/4 | 0/4 | 0/4 | 0/4 | 3/4 | 1/1 | 0/2 | 2/2 |
| VII (3536) | 0/4 | 0/4 | 0/4 | 0/4 | 2/4 | 2/4 | 1/2 | 1/1 |
| VIII (4333) | 2/4 | 4/4 | 3/3 | 1/2 | 4/4 | 2/2 | 0/2 | 2/2 |
| IX (5099) | 1/4 | 0/4 | 0/3 | 0/2 | 3/4 | 1/2 | 0/2 | 1/2 |
| X (8053) | 0/4 | 3/4 | 1/3 | 1/3 | 2/4 | 1/1 | 0/2 | 2/2 |

*Numerator indicates the number of animals with positive findings.
Denominator indicates the number of animals tested.

*Cross Reaction Experiments in Rabbits*

This was an attempt to determine whether the chromogenic extracts were antigenically related to each other and whether some antigenic relationship could be demonstrated between them and human or avian tuberculins. Two strains were selected: No. III, which had elicited lesions in rats, hamsters, guinea pigs, and No. VII, which had produced lesions in the same animal and species and also in one hen. For each strain, two rabbits approximately 2 kg. in weight were inoculated with $13 \times 10^8$ organisms, one animal by the intravenous route, the other intraperitoneally. Skin tests were made 5 weeks later with the homologous and heterologous chromogenic extracts, human O.T., avian tuberculin, and a control consisting of Sauton's medium reduced to 1/10th of its volume.

Rabbits infected with strain III showed: no reactions with avian tuberculin or human O.T.; positive reactions with homologous strain in both rabbits; no reactions with extracts of strains V and IX, and positive reactions with extracts of strains I, II, IV, VI, VII, VIII, X.

Rabbits infected with strain VII showed: no reactions with avian tuberculin or human O.T.; positive reactions with homologous strain in both rabbits; doubtful reactions with extracts of strains I, II, III, IV, V, VI, and VIII and Sauton control, and negative reactions with extracts of IX, X.

From the results obtained with strain III, it seemed obvious that antigenic relations existed between this strain and seven others, but unfortunately the results with strain VII did not confirm this finding. As no further cross reaction experiments have been carried out, no convincing data on the antigenic patterns of these strains are available.

## Conclusions

The 10-strains of chromogenic acid- and alcohol-fast rods that we have examined for their morphological, cultural, and biological characters differ in one or more respects from the species of *Nocardia* and *Mycobacterium* with which they have been compared. We consider, however, that the sum total of the characteristics of these strains justifies their inclusion in the genus *Mycobacterium*. The fact that they were isolated in pure culture from *closed* tuberculoid lesions of cervical lymph nodes in children suggests that they may be a distinct species of *Mycobacterium* and must be considered the actual cause of the lymphadenitis. This distinction is reinforced by the results of infection of animals, by the apparent specificity of the induced allergic response, and by the distinguishing characteristics noted in cultural studies. The most distinctive cultural characteristics are the slow-growing yellow-pigmented colonies, which are easily emulsified in saline, and the ability of the organism to utilize paraffin as a sole source of carbon.

The name *Mycobacterium* ***scrofulaceum*** (n.sp.) is proposed.

## Diagnostic Description*

*Morphology and staining reactions.*—Short rods when found on direct smear from lesions; granular, often filamentous rods on litmus milk medium; cords inconstant (Dubos' medium); no branching demonstrated; nonmotile. Acid–alcohol fast, resisting 10 minutes' exposure to acid and alcohol; Gram-positive or partially decolorized, this emphasizing the beading.

*Cultural characteristics.*—Bright yellow colonies appearing on Loewenstein's medium 4 to 5 weeks following inoculation, 1 to 2 mm. in diameter, smooth, convex, opaque, and emulsifying readily in normal saline to give homogenous suspensions. Growth on streak subcultures appears in 10 to 14 days.

*Optimum temperature and oxygen requirement.*—Optimum 37° C.; strict aerobe.

*Physiology.*—Optimum growth on Dorset's egg medium, Bordet-Gengou medium, and Dubos' fluid medium; usually utilizes paraffin as a sole source of carbon; no acid produced from any of the carbohydrates tested. Indol not formed. Gelatin not liquefied. Nitrates not reduced to nitrites. No phenol tolerance. Neutral red test negative.

*Pathogenicity.*—Localized lesions produced in guinea pigs inoculated by intraperitoneal route.

*Source.*—From cervical adenitis in children.

## Acknowledgments

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# NOTES

## LEUCOCIDAL EFFECT OF STAPHYLOCOCCAL δ-LYSIN

A. W. Jackson and R. M. Little

The formation of leucocidal substances (leucocidin) by *Micrococcus pyogenes* var. *aureus* has been recognized since 1894 when Van de Velde (9) observed the destruction of rabbit leucocytes by the products of some strains of this microorganism. Shortly afterwards Neisser and Wechsberg (6) observed that the ability of rabbit polymorphonuclear leucocytes to reduce methylene blue anaerobically was inhibited by leucocidin. In 1936, Wright (10) found that the product causing inhibition of methylene-blue reduction is identical with α-toxin. Existence of a second leucocidin was demonstrated by Valentine (8) when cultures were grown on solid media under an atmosphere of 20% carbon dioxide in oxygen.

Since the conditions found necessary to produce the second leucocidin (8) are identical with those required for the formation of δ-lysin (5), it seemed possible that δ-lysin may be identical with the second leucocidin. It was of interest therefore to conduct experiments to determine the validity of this hypothesis. The possession of leucocidal activity by δ-lysin of *M. pyogenes* var. *aureus* was reported recently (2) but it is desirable to record briefly the experiments from which this conclusion was drawn.

Before presenting our observations it should be noted that Valentine (8) found the second leucocidin caused disruption of human polymorphonuclear leucocytes, whereas the leucocidin identical with α-toxin causes a disorganization of internal structure and swelling of the leucocytes, but bursting does not occur.

δ-Lysin was produced from selected cultures[1] by a modification (2) of the method employed by Marks and Vaughan (5). The lysin was partially purified by solvent precipitation in the cold (2) but contained some α-toxin. The same cultures were grown also in the above medium without agar, under which conditions it has been shown (5) that α-toxin is produced but δ-lysin is not. Tests applied as described elsewhere (3) showed α-toxin prepared in this manner to be free of δ-lysin.

Typical results of tests performed to determine the type of leucocidal activity possessed by each of the above preparations are shown in the accompanying table. The human leucocytes were obtained by differential sedimentation (1) from freshly drawn blood and washed in modified Hanks' solution (4) to which traces of acetate and ascorbic acid were added (7).

It will be seen that the typical effect of the second or "human" leucocidin of Valentine (8) is obtained only from cultures grown on solid medium.

[1]Cultures employed: Laboratory of Hygiene's Nos. 1363, 2428, 3133.

LEUCOCIDAL EFFECTS OF δ-LYSIN

| Lysin* | Condition of leucocytes† | | |
|---|---|---|---|
| | Normal | Enlarged | Disrupted |
| Surface culture | | | |
| δ | | | + |
| δ + α − antitoxin | | | + |
| δ heated 60°, 30 min. | | | + |
| Liquid culture | | | |
| α | | + | |
| α + α − antitoxin | + | | |
| Control | + | | |

*Lysins were employed at concentrations approximately 10 times those required to hemolyze a 1% suspension of the appropriate erythrocytes. Duration of exposure of leucocytes to lysin, 20 minutes at 37° C.

†Smears examined microscopically after being stained with Wright's stain. Examination of a number of smears indicated that where changes occurred all the polymorphonuclear leucocytes present were affected by the lysins at the concentration employed.

Moreover, its effects are not inhibited by α-antitoxin, whereas the product obtained from liquid cultures causes the type of leucocyte damage associated with α-toxin and is completely inhibited by α-antitoxin. In addition to the above results, it can now be reported that leucocidal activity typical of δ-lysin is still possessed by crude preparations of this lysin after the preparations are heated at 60° for 30 minutes to destroy the α-toxin originally present.

The above data support the contention that δ-lysin may be identical with the second leucocidin of Valentine (8) and consequently this lysin, which apparently does not possess lethal properties (5), may none the less cause some damage to leucocytes during staphylococcal infections. Final proof, however, must await more complete purification of the lysin.

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